ALKALINE PHOSPHATASE IN AMELOGENESIS[1]

GERRIT BEVELANDER AND P. L. JOHNSON

Departments of Histology, College of Dentistry, and The Graduate School of Arts and Science, New York University

ELEVEN FIGURES

INTRODUCTION

In the several studies which have been reported concerning the histochemical localization of alkaline phosphatase in the developing tooth, considerable disagreement exists among various investigators. This is especially true in regard to the ameloblasts, the cells which are intimately associated with the process of enamel formation. On the one hand, Kabat and Furth ('41), Engel and Furuta ('42) and Gomori ('43) assert that ameloblasts do not contain phosphatase. Other observers, however, Horowitz ('42), Bevelander and Johnson ('45), and Greep, Fischer and Morse ('48), maintain that ameloblasts do contain the enzyme.

In view of the diversity of opinion concerning the presence of this enzyme in the ameloblasts, we decided to investigate this problem again and extend our previous observations to include the entire developmental and functional span of these cells. By extending our observations to encompass not only the very young pre-calcification stages, or isolated developmental stages as previous investigators have done, we ascertained first the phosphatase localization during the histogenesis of the ameloblast, and second observed whether this enzyme was present in these cells during matrix formation and subsequent calcification.

[1] This work was aided in part by a grant made available by the U. S. Public Health Service.

THE ANATOMICAL RECORD, VOL. 104, NO. 2
JUNE, 1949

MATERIAL AND METHODS

The teeth used in this study were obtained from fresh pig embryos ranging in size from 115 mm to 210 mm (eruption). The deciduous canine was used throughout in these observations. In addition, the incisors of the rat were also tested.

Tissues were fixed in cold 95% alcohol. Alkaline phosphatase was localized according to the method of Gomori ('39, '41) with slight modifications. All stock solutions were freshly prepared and kept in the refrigerator. The hydrogen-ion concentrations were checked before and after incubation. Paraffin of 52° melting point was used and embedding time was kept to a minimum by employing vacuum embedding methods. Sections were prepared using undecalcified tissues as well as decalcified tissues. The latter were treated by the several decalcification methods described by Shipley ('39), Lorch ('47), and Greep, Fischer and Morse ('48).

We wish to point out in connection with the choice of our material that the canine tooth in the 120 mm pig embryo exhibits all of the developmental stages of the ameloblast up to and including pre-enamel formation. Intermediate, and complete development of coronal enamel matrix [2] takes place by the time the embryo reaches 210 mm. By the judicious choice of material we were able to study the entire developmental history of the ameloblasts, the formation of enamel matrix,[2] the subsequent calcification of enamel, and to correlate this development with phosphatase localization in the ameloblasts and other tissues in the pigs' teeth as well as the incisor of the rat.

OBSERVATIONS

Figure 1, is a photograph of a canine tooth of a 115 mm pig embryo, showing the general pattern of phosphatase distribution in the several tissues of the developing tooth. Examination of this figure shows that the enzyme is most highly

[2] Enamel matrix refers to embryonic enamel which is calcified to the extent of about 30% of the fully matured tissue.

concentrated in the coronal region of the pulp, odontoblast cytoplasm and nuclei, and the combined epithelium. In this stage of development the ameloblasts are essentially negative except for the nuclei.

One may also observe in this photograph that the formation of calcified dentin has already taken place in the coronal region of the tooth. In the middle one-third of the tooth, odontoblasts have differentiated and are phosphatase positive. In this region however, dentin has not as yet undergone calcification.

A short time before enamel matrix begins to be laid down the ameloblasts are phosphatase positive in the cytoplasm as well as the nuclei. From this time on, the enzyme is present in these cells during the time that matrix formation takes place as shown in figures 2, 4, 6 and 7, and also in the later development of this tissue when the final calcification occurs as shown in figures 3 and 8. The coronal region of the crown of those teeth in which the enamel had been fully calcified (just prior to eruption) shows a gradual reduction in phosphatase content of the ameloblasts as shown in figures 5, 8 and 9.

Examination of the pig material indicates that phosphatase is localized in the ameloblasts shortly before enamel formation takes place. The enzyme is present during matrix formation and also during subsequent calcification. As the tooth begins to erupt the enzyme gradually disappears from the ameloblasts as they begin to undergo regressive changes.

A study of phosphatase localization in the ameloblast of the erupted rat's incisor shows a distribution of the enzyme in this species essentially the same as that observed in the several developmental stages of the pigs' teeth which we examined. As in the case of the pigs' canine, the enzyme is present in the nucleus and cytoplasm of the ameloblasts before enamel formation begins. During the time matrix is forming (fig. 4) and subsequent calcification (fig. 3) the enzyme is localized throughout the cell. When calcification

is complete, phosphatase is gradually reduced, first in the cytoplasm (fig. 5) and eventually in the nucleus.

Decalcification and reactivation. The several decalcification methods which have been proposed in connection with the demonstration of alkaline phosphatase in calcified tissues was undertaken and all of the methods previously mentioned yielded a positive phosphatase reaction in the tissues we studied. Some of these preparations are shown in figures 3, 7, 10 and 11. While it appears to be true that phosphatase can be demonstrated in tissues which are subjected to buffered organic acid solutions, the several methods in our experience have certain disadvantages, viz.; prolonged decalcification (more than 24 hours) decidedly reduces the intensity of the reaction. There appears to be an uneveness or spottiness in the distribution of the enzyme and finally the cells take on a rather granular appearance. (Compare figs. 2, 4 and 6, undecalcified sections with figs. 3, 7 and 10, decalcified preparations.)

Tests for diverse phosphatases. The usual methods for ascertaining the presence of diverse enzyme systems in tissues is to test them under conditions in which the substrate and the acidity (pH) of the incubating solutions are varied, and also to observe the effect of enzyme inhibitors or accelerators.

The enzyme localization in the ameloblasts, and incidentally other dental tissues, were subjected to incubation procedures using five different substrates whch yielded phosphatase activity with respect to the substrates employed as follows: nucleic acid > fructose diphosphate > sodium glycerophosphate > sodium diphenylphosphate > adenylic acid.

Visualization of phosphatase was also tested for each of the 5 substrates mentioned above at pH values ranging from 5–11. The maximum activity occurred at 9–9.5, fairly intense localization at 10, while at 11 the tissues were practically negative. On the acid side of the range, activity appears practically negligible below pH 8. In all of the various pH tests which were made, the enzyme in the nuclei appears to

withstand variations in the pH of the incubating solutions without losing activity somewhat better than the cytoplasm.

Activators and inhibitors. The use of various substances reported to affect the activity of alkaline phosphatase was tested for each substrate mentioned above at concentrations of M .01–.001. Mg^{++}, urea and thiourea consistently enhanced the activity somewhat. Inhibition occurred consistently upon the addition of Zn, glycine, NaS, iodoacetic acid, and ascorbic acid. Cyanide and Fl appeared to have little or no effect on the activity.

As a result of the several tests utilizing diverse substrates, pH, and several accelerators and inhibitors, we were unable to ascertain any evidence which might indicate the presence of diverse phosphatases (phosphomonoesterases) in the dental tissues.

DISCUSSION

In our introductory remarks we stated that the localization of alkaline phosphatase in ameloblasts is subject to diverse opinion. One opinion held is that ameloblasts are negative with respect to the enzyme. This contention, it should be noted, is based upon limited observations of a very incomplete developmental series. Since the studies reported by us, Bevelander and Johnson ('45), in which we described the presence of phosphatase in ameloblasts, Greep, Fischer and Morse ('48) have also reported phosphatase positive ameloblasts in the molars of the rat.

Examination of the material described in the present study shows that phosphatase is present in the nuclei and cytoplasm of the ameloblasts before enamel matrix formation takes place. Just before and during the elaboration of the matrix a high concentration of phosphatase is maintained. While the matrix is being elaborated, partial calcification of this tissue also takes place. When calcification of the matrix is complete, phosphatase in the ameloblasts gradually disappears.

One of the features in connection with the elaboration of enamel which we though might throw some light on the

reported correlation between the presence of phosphatase and calcification is the rather unique manner in which this tissue is formed. The embryonic enamel (matrix) is composed of a considerable amount of organic substance (keratin), fluid, and some mineral salts. Parenthetically it might be added that evidence at hand indicates that the proportion of phosphate to calcium in this state of development is much higher than in the fully calcified enamel. The subsequent development of enamel consists in the deposition of mineral salts.

If phosphatase were concerned primarily with the formation of the organic constituents of enamel which roughly correspond to the fibrous components of bone, one might expect that when the enamel matrix was completed the enzyme would disappear from the ameloblasts and other epithelial structures making up the enamel organ.

This condition however, does not hold, as shown by our own studies, as well as those of Greep, Fischer and Morse ('48). Specific evidence indicating that phosphatase is responsible for the deposition of mineral salts is likewise not confirmed. From this and our previous studies as well as the several reports dealing with phosphatase and calcification, one can only deduce that alkaline phosphatase is present (Moog, '44), during the period when differentiation or elaboration of several diverse tissues or substances occur. In other words, phosphorylation appears to be a process which takes place during the histogenesis and differentiation of several tissues and organs and is not limited to any specific metabolic process such as the deposition of mineral salts in calcified tissues.

Localization of phosphatase in tissues which are first subjected to decalcifying fluids of various composition has according to our experience a number of serious disadvantages. The most important one appears to be that the histochemical visualization of the enzyme is modified or completely lost. In our opinion results obtained following these methods must be evaluated with extreme caution.

In regard to the presence of diverse alkaline phosphatases in the dental tissues our preparations did show slight differences in intensity of enzyme localization with different substrates, but in no case any particular specificity of localization in either different tissues or different regions of the cell.

In this respect, our observations as far as dental tissues are concerned, are in complete agreement with the results obtained by Gomori ('49) who has studied the problem of substrate specificity extensively, and who has also pointed out the inherent sources of error and difficulties which beset the determination of diverse enzymes in histochemical preparations.

SUMMARY

1. The localization of alkaline phosphatase has been ascertained in the ameloblast of the developing tooth.

2. The ameloblasts become phosphatase positive just before enamel matrix formation occurs.

3. These cells retain the enzyme during matrix formation and the subsequent calcification of the enamel.

4. Various tests designed to elicit the presence of diverse alkaline phosphatases in the several dental tissues were completely negative.

LITERATURE CITED

BEVELANDER, G., AND P. L. JOHNSON 1945 The histochemical localization of alkaline phosphatase in the developing tooth. J. Cell. and Comp. Physiol., *26:* 25–33.

ENGEL, M. B., AND FURUTA, W. 1942 Histochemical studies of phosphatase distribution in developing teeth of albino rats. Pro. Soc. Exp. Biol. and Med., *50:* 5–9.

GOMORI, G. 1939 Microtechnical demonstration of phosphatase in tissue sections. Proc. Soc. Exp. Biol. and Med., *42:* 23–26.

———— 1941 Distribution of phosphatase in normal organs and tissues. J. Cell. and Comp. Physiol., *17:* 71–83.

———— 1943 Calcification and phosphatase. Am. J. Pathol., *19:* 197–207.

———— 1949 Histochemical specificity of phosphatases. Proc. Soc. Exp. Biol. and Med., *70:* 7–11.

GREEP, R. O., C. FISCHER, AND A. MORSE 1948 Alkaline phosphatase in odontogenesis and osteogenesis and its histochemical demonstration after demineralization. J. Am. Dent. Assoc., *36:* 427–442.

HOROWITZ, N. 1942 Histochemical study of phosphatase and glycogen in fetal heads. J. Dent. Res., *6:* 519–527.

KABAT, E. A., AND FURTH, J. 1941 A histochemical study of the distribution of alkaline phosphatase in various normal and neoplastic tissues. Am. J. Pathol., *17:* 303–318.

LORCH, I. J. 1947 Localization of alkaline phosphatase in mammalian bones. Quart. J. Micro. Sci., *88:* 367.

MOOG, F. 1944 Localization of alkaline phosphatase and acid phosphatase in the early embryogenesis of the chick. Biol. Bull., *86:* 51–80.

SHIPLEY, P. G. 1939 Decalcification with magnesium citrate. Handbook of Microscopical Technique (p. 348). C. E. McClung. Paul Hoeber, Philadelphia.

PLATE 1

EXPLANATION OF FIGURES

1 Orientation section of the canine tooth and associated tissues of a 115 mm pig embryo. Note the highly reactive pulp and odontoblasts, less reactive undifferentiated ameloblasts and highly reactive combined epithelium and stellate reticulum. × 125.

2 Photograph of fully differentiated functionally active, secreting ameloblasts of a 140 mm pig incisor. Note the thickness of the enamel matrix; reduced epithelium and ameloblasts are highly reactive. × 350.

3 Photograph of differentiated ameloblasts of 6-day rat's incisor in region of calcified enamel. (Decalcified by Lorch's method.) × 350.

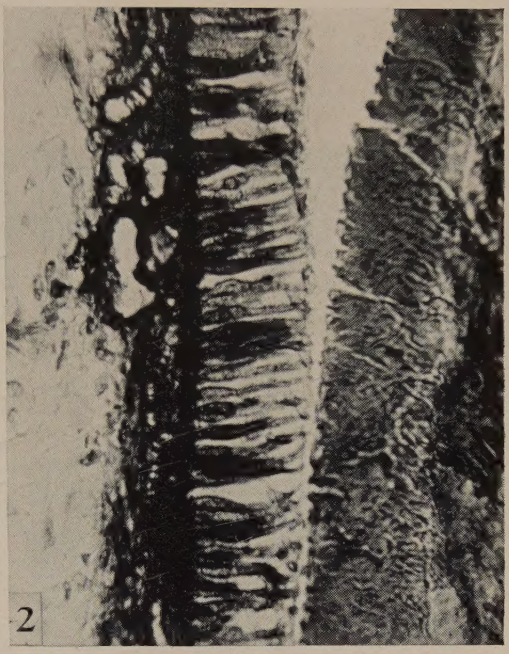
4 Phosphatase positive ameloblasts of a 6-day rat incisor, some enamel matrix is present, abundant dentin. × 350.

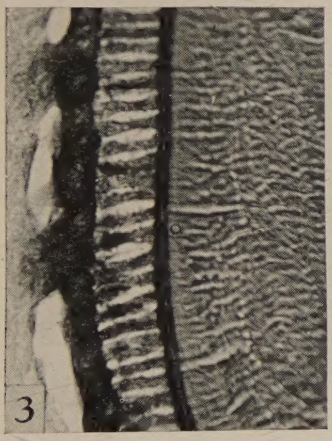
5 Cervical region of incisor tooth of 6-day rat. Ameloblastic nuclei are positive, but cytoplasm is negative. Area about Tomes' processes are highly positive. Considerable enamel and dentin present. × 350.

6 Cross section of incisor of three-day rat showing enamel matrix. Note highly reactive ameloblasts. × 700.

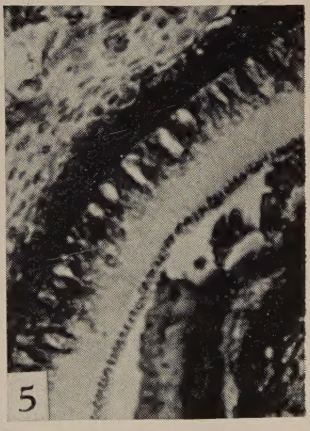
7 Section of enamel matrix and ameloblasts of 170 mm pig canine. Note highly reactive ameloblastic nuclei and cytoplasm. (Decalcified by Kramer and Shipley's method.) × 700.

1


2


3

4


5

6

7

PLATE 2

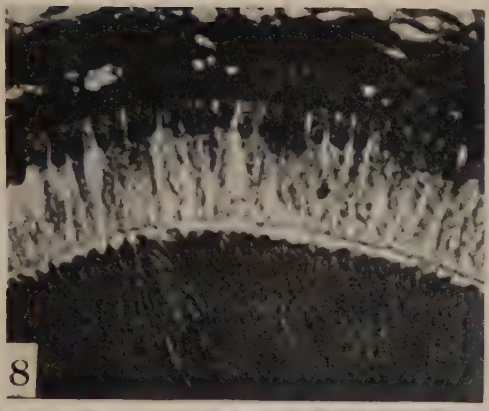
EXPLANATION OF FIGURES

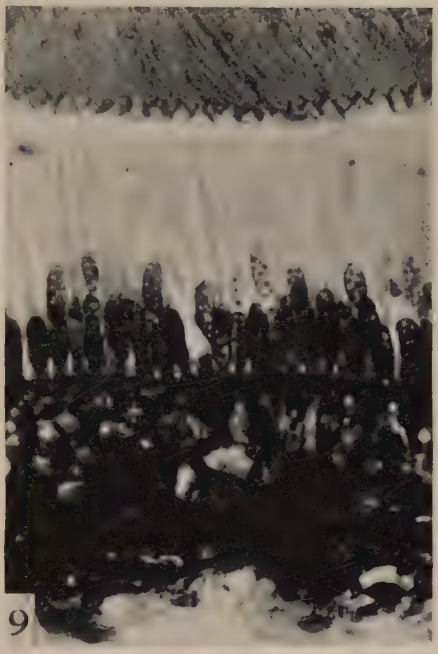
8 Labial section at cuspal end of canine tooth of 170 mm pig. The cytoplasm of the ameloblasts at this stage is nearly depleted of phosphatase. × 700.

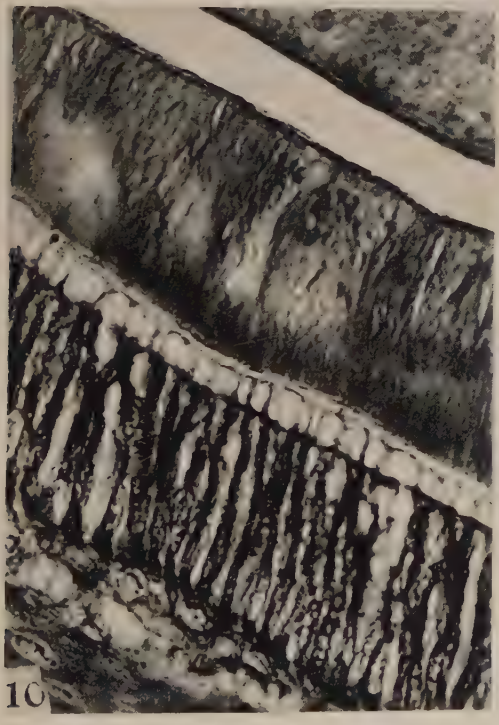
9 Section of cusp of canine tooth of 170 mm pig toward lingual surface. Combined epithelium and nuclei of ameloblasts phosphatase positive. Cytoplasm of ameloblasts essentially negative. × 1050.

10 Section of cervical region of 170 mm pig canine showing enamel matrix and ameloblasts. (Decalcified by Kramer and Shipley's method.) × 1050.

11 Oil immersion photo of same section shown in figure 10. × 1500.


8


9


10

11

VENOUS ANOMALIES IN A DOG

I. ABSENCE OF THE PORTAL VEIN

II. CONTINUITY OF LOWER PART OF INFERIOR VENA CAVA WITH THE AZYGOS VEIN

JOHN HICKMAN, M.R.C.V.S.,
Fellow of the Animal Health Trust, London, England,
Assigned to the Mayo Foundation,

JESSE E. EDWARDS, M.D.,
Section on Pathologic Anatomy, Mayo Clinic.

FRANK C. MANN, M.D.,
Division of Experimental Medicine, Mayo Foundation,
Rochester, Minnesota

SIX FIGURES

The subject of this report was a mongrel bitch, aged 10 months, which had died from bronchopneumonia following canine distemper.

The case is being reported for two reasons: first, to place on record an unusual type of congenital venous anomaly; and second, because the dog had, as far as is known, lived a normal life unhampered by its vascular malformations.

POST-MORTEM EXAMINATION

At necropsy the inferior vena cava was found to be formed by the confluence of the right and left common iliac veins at the level of the 5th lumbar vertebra. It passed cranially on the ventral surface of the psoas minor muscle, to the right of the abdominal aorta, and at the first lumbar vertebra received the renal veins (figs. 1 to 3).

From this point the vessel, instead of continuing the normal course of the inferior vena cava by separating from the

aorta and passing forward between the right crus of the diaphragm and the pancreas, followed the normal course of the azygos vein and entered the superior vena cava just above the right atrium (figs. 1 to 3). The azygos vein was greatly dilated and approximated the expected diameter of the inferior vena cava.

There was no portal vein (fig. 2); the gastrosplenic, pancreatic and ileocecocolic veins emptied, as three separate

Fig. 1 Right lateral view of the torso. Above the kidneys the inferior vena cava blends imperceptibly with the azygos vein.

Fig. 2 The upper portion of the torso. The azygos vein, into which the inferior vena cava passes, is wide. It joins the superior vena cava (S. V. C.) just before the latter enters the right atrium. Leaving the liver is a narrow vein (C. H. V.) which, after piercing the diaphragm, enters the right atrium. It is the common hepatic vein. The right lung has been removed. Its hilum lies just below the terminal portion of the azygos vein. C.T., the common venous trunk formed by the union of the abdominal visceral veins. This trunk enters the systemic venous system at the imaginary point of junction between the inferior vena cava and the azygos vein. K., right kidney.

tributaries, into a short venous trunk. At the imaginary point of junction between the inferior vena cava and the azygos vein this venous trunk then joined the major systemic vein (figs. 4 and 5).

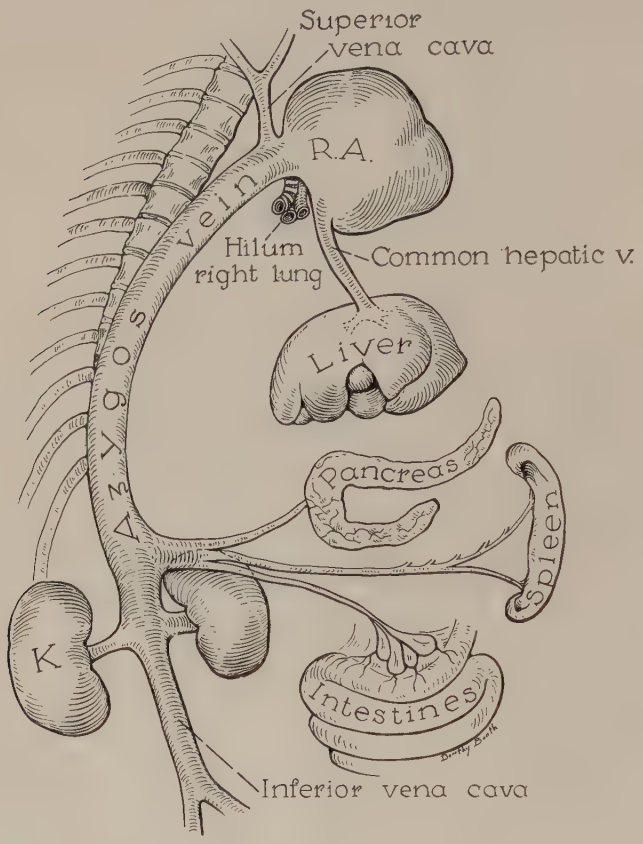


Fig. 3 Diagrammatic representation of the venous pattern shown in figures 1 and 2. K., right kidney. R. A., right atrium.

Dissection of the visceral surface of the liver revealed only the hepatic artery and the common bile duct, both of which were normal in structure and anatomic arrangement. No vein comparable to a portal vein entered the liver (fig. 5*b*).

A narrow vein emerged from the parietal surface of the liver. This vessel followed the course taken by the normal

Fig. 4 Details of the vascular structures in the suprarenal region. *a*. The common venous trunk (C. T.) is shown to be formed from the pancreatic vein and from the ileocecocolic vein. Only a portion of the gastrosplenic vein, which also contributes to the formation of the common venous trunk, is shown in this illustration. A., aorta. The vessel arising at the level of the letter "A" is the hepatic artery. *b*. The common venous trunk (C. T.) joins the systemic vein at the junction of the inferior vena cava and the azygos vein.

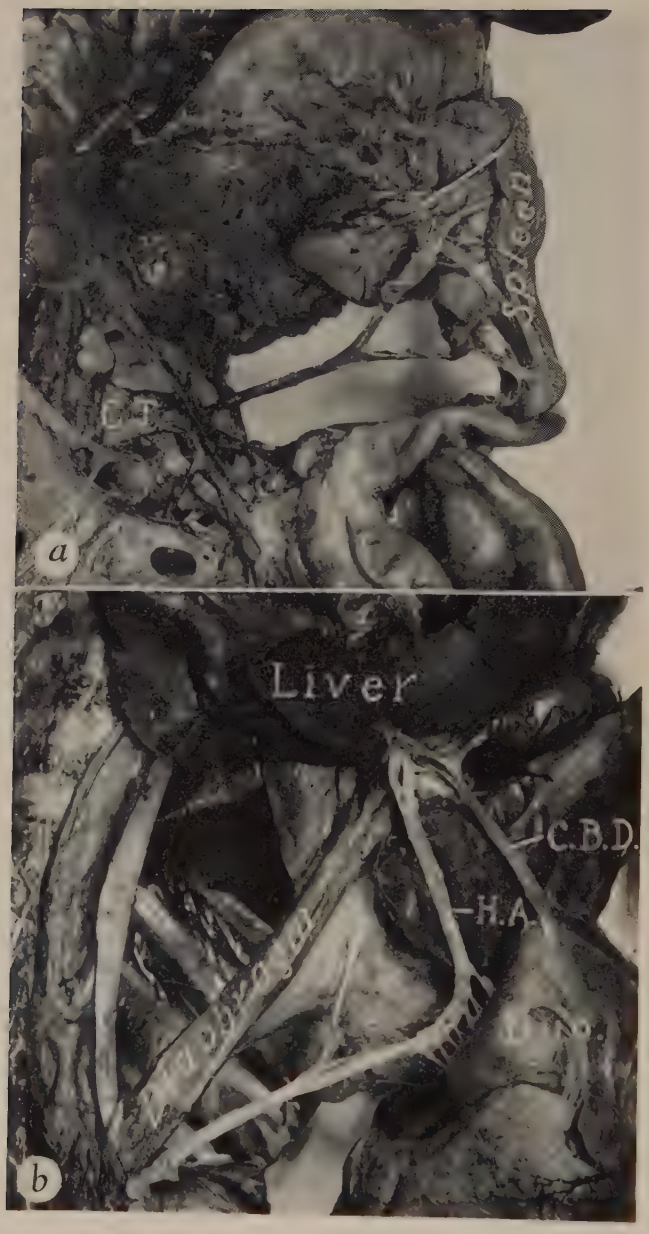


Fig. 5 *a*. The transverse colon and pancreas have been reflected upward so that now the pancreatic vein runs more or less vertically into the common venous trunk (C. T.). The gastrosplenic vein is shown to enter the common venous trunk. *b*. The visceral aspect of the liver and adjacent structures. The only blood supply to the liver is by way of the hepatic artery (H. A.). No portal vein enters the liver. The common bile duct (C. B. D.) runs from the junction of the cystic and common hepatic ducts into the duodenum.

inferior vena cava after it leaves the liver; it passed through the foramen venae cavae of the diaphragm and opened into the posterior part of the right atrium. This vein was, in effect, a common hepatic vein (figs. 2 and 3). There were no anomalies of the heart.

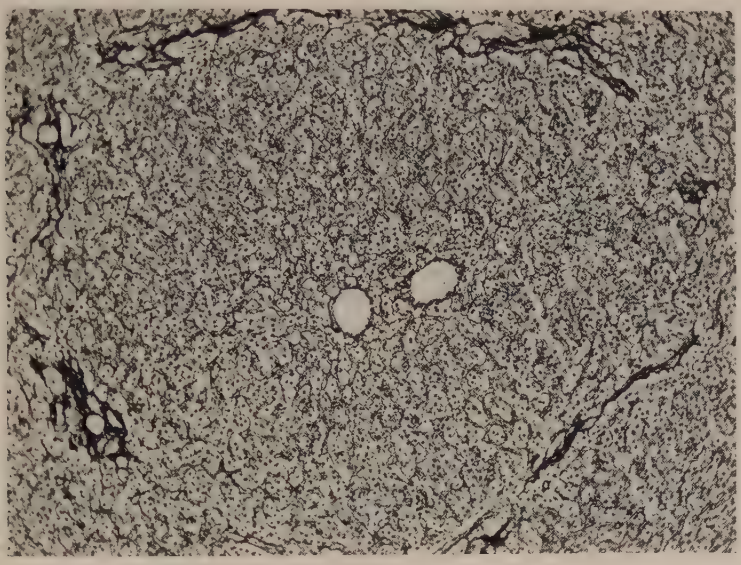


Fig. 6 Liver (Gomori's stain for reticulum × 90).

HISTOLOGY OF THE LIVER

The histologic structure of the liver appeared normal in most respects (fig. 6). The characteristic hepatic lobular arrangement was less distinct in some regions than is usually noted in the liver of the dog. This appeared to be owing to a failure to delineate a central vein with radiating trabeculae. In other regions the site usually occupied by the central vein appeared to contain a slightly increased amount of connective tissue. There was considerable congestion between the groups of hepatic cells and in some regions there was also a large number of large bacilli. The latter appeared similar to the anaerobic bacillus (Wolbach) which is found

so frequently in cultures of hepatic tissue of the dog or after occlusion of the arterial blood supply to the liver of the dog. The great number of bacilli was probably explainable on a basis of post-morten growth.

COMMENT

It is evident that two separate anomalies may be distinguished in this case:

1. The continuity of the lower inferior vena cava with an enlarged azygos vein is one malformation. Although rare, it has been described in man (Griffith, 1891; Miller, '25).

2. The second malformation, which is of greater interest, was characterized by absence of the portal vein in the usual sense. Instead, all the great abdominal visceral veins joined to form a common venous trunk, which in turn communicated with the major systemic vein of the abdomen. This provided the liver with only one source of blood supply, the hepatic artery.

It is interesting to note that this anomaly resembles what might be termed a naturally occurring true Eck fistula. The only difference is that, in the anomaly, the blood from the abdominal organs entered the right atrium via the azygos vein, whereas, in a true Eck fistula, the portal blood is so shunted as to enter the right atrium via the inferior vena cava.

Venous anomalies essentially identical to those herein described by us in a dog were described in man by Abernethy (1793). His patient was a female child whose estimated age at the time of death was 10 months. In his case there was situs inversus of the thoracic organs. The liver was centered at the midline. Abernethy stated that a portal vein was formed in a normal manner but instead of entering the liver, this vein joined the inferior vena cava, at the level of the renal veins. Above this, the inferior vena cava had the course of an azygos vein and joined the superior vena cava as the latter lay in the right side of the body. Abernethy stated further that beyond this the "conjoined vein passed

beneath the basis of the heart, to expand into the anterior (left sided, venous) auricle." It seems likely to us, since there was situs inversus of the thoracic organs and since the superior vena cava was on the right side, that the superior vena cava joined the coronary sinus on the right side of the inverted heart. This interpretation would be consistent both with the illustrations published by Abernethy and with that part of his description which has just been quoted. In any event the course of blood in the superior vena cava and in the azygos vein would, as in our case, be into the venous atrium. As in our case, the veins leaving the liver joined to form a narrow trunk which pierced the diaphragm (on the left side in Abernethy's case) and joined the venous atrium at the expected location of the inferior vena cava. The blood supply to the liver was exclusively arterial by way of a wide hepatic artery.

Kiernan (1833), described the case of Wilson, which he had had the opportunity of studying personally. Wilson's patient was a girl, aged 13 years, who had died as a result of an accidental injury to her head. In that case the portal vein did not enter the liver but instead terminated in the inferior vena cava. An accessory splenic vein also joined the inferior vena cava. This case varied from Abernethy's and from ours in that the course of the inferior vena cava was evidently normal. Nevertheless, as in our case, and in Abernethy's, the liver was supplied with blood exclusively by the hepatic artery. In Wilson's case this artery was wider than normal and it arose as an independent branch of the abdominal aorta. The lumen of the umbilical vein was found to be obliterated.

In the description of our case we have avoided calling the common venous trunk formed by union of the abdominal visceral veins the portal vein, since this vein, by avoiding the liver, does not fulfill the function of a portal vein and it therefore seems to us appropriate to avoid referring to it as such anatomically.

SUMMARY

Venous anomalies in a dog have been described. These may be summarized as follows:

1. The lower portion of the inferior vena cava and the azygos vein were confluent.
2. The portal vein was absent. The blood from the abdominal organs collected into a common venous trunk which in turn drained into the major systemic vein of the abdomen.
3. The inferior vena cava above the liver was narrow and was merely a common hepatic vein.
4. Blood to the liver was supplied solely by the hepatic artery.
5. There had evidently been no disturbance of hepatic function resulting from the anomalous blood supply to the liver.

REFERENCES

ABERNETHY, JOHN 1793 Account of two instances of uncommon formation in the viscera of the human body. Phil. Tr. Roy. Soc. London, *83:* 295–299.

GRIFFITH, T. W. 1891 A case of transposition of the thoracic and abdominal viscera with congenital malformation of the heart and certain abnormalities of the arterial and venous systems. J. Anat. and Phys., *26:* 117–129.

KIERNAN, FRANCIS 1833 The anatomy and physiology of the liver. Phil. Tr. Roy. Soc. London, *123:* 711–770.

MILLER, A. J. 1925 Congenital heart disease with partial situs inversus, absence of inferior vena cava and other anomalies. Am. J. Path., *1:* 467–476.

SOME RESPONSES OF LIVING BLOOD VESSELS AND CONNECTIVE TISSUE TO TESTICULAR GRAFTS IN RABBITS[1]

ROY G. WILLIAMS

Department of Anatomy, School of Medicine, University of Pennsylvania, Philadelphia

SIX FIGURES

In studying the microscopic appearance and behavior of grafted testicular components with the transparent chamber technique, it was observed that the blood vessels in and around grafts containing interstitial cells grew and arranged themselves quite differently from those around grafts in which interstitial cells were missing. Similar vascularization has not been seen in any other type of tissue grafted to transparent chambers. It was considered likely that the unusual vascular response was related to a spreading factor which the testis is known to produce. The vascular characteristics in and around grafts containing interstitial cells were similar to those in certain simple hemangiomas. If the two were related, that is, the vascular response and a spreading factor, then a similar mechanism might have a bearing on the genesis of some kinds of hemangiomas. This account is given as a preliminary to an investigation of the latter possibility. It deals with the growth and behavior over a long period of living vessels in and around autogenous testicular grafts.

[1] This work was supported by grants from three sources: (1) The Cancer Research Fund of the University of Pennsylvania established by a gift from Anna May Conway and a bequest of Louise M. Holroyd; (2) The American Cancer Society; and (3) The Faculty Research Fund.

METHODS

A variation (Williams, '48) of the transparent chamber method of Sandison and Clark was used. A chamber made of tantalum, glass and mica was installed in the left ears of 4 unrelated rabbits each about 6 months old. The device has a central thin, transparent region into which blood vessels and connective tissue grow.

After vascularization of a chamber the mica cover slip was removed. Small autogenous grafts of testes were then made directly on the thin layer of vessels and the cover replaced. The improved chamber used produces very little, if any, foreign body reaction and cannot be extruded. Therefore, microscopic and other types of studies may be carried out for an indefinite period in the lifetime of the animal. So far, studies have been made daily or at frequent intervals for 10 months and are being continued.

Grafts were obtained by incising the scrotum and tunica albuginea. A good sized piece of tubular tissue was removed and placed at room temperature in a shallow dish of Tyrode solution. Under a dissecting microscope pieces for grafting were prepared with instruments similar to those used in experimental embryology. Attempts were made to have separate grafts composed of interstitial cells without tubular cells, tubules without interstitial cells and others of interstitial cells and pieces of tubule combined. This is an uncertain procedure but if the grafts are sufficiently small and numerous the result frequently turns out as intended.

In an additional animal and in two of the 4 with chambers in their left ears numerous testicular grafts were made between skin and cartilage in the intact right ear. They could be located through the intact skin and removed individually at various times without disturbing the remaining grafts. When fixed and sectioned they served as a check on conditions observed in living grafts. Some were left in place and used to determine the spreading reaction around the graft.

OBSERVATIONS

All grafts in the 4 chambers survived. One animal died of intestinal obstruction after 4 months and another died of pneumonia after 6 months. The two remaining animals are in good health and the chambers in excellent condition 10 months after their installation.

Around grafts composed only of tubules capillaries formed, as they generally do, by sprouting. Vessels did not invade the tubules but formed plexuses of coarse mesh around them. These vessels were similar in all aspects of their morphology and behavior to those in nongraft regions (fig. 5). Once established the plexuses underwent no signficant changes irrespective of changes which the tubules might be undergoing. But, in and around grafts composed of interstitial cells only and of interstitial cells plus tubules the vascular response was quite different. Within 36 hours large, noncirculating blood-filled sinusoids were observed at the margins of the grafts and within them. The rapid appearance of such large vessels suggested that the original vessels of the graft might have persisted, sealed themselves off and made connections with those of the host region. However, they were not observed to do so and the statement only serves to emphasize the speed with which vascularization began. Vessels continued to form in and over the grafts and also for as much as 100 μ beyond the borders of the grafts. This latter response was unusual since in no other of the several types of tissue previously studied with the transparent chamber method was the number or pattern of vessels materially affected in the host region beyond the grafts.

When compared with thick cleared sections of whole testis in which the vessels were injected with India ink (fig. 6), the degree of vascularity of the grafts appeared to be greater than in normal testis and the graft vessels were different in other ways.

In general, the circulation of testicular grafts with interstitial cells was predominantly venous (fig. 3). In other types of transplants the vessels have been composed of arterioles,

capillaries and venules (fig. 4). In testis grafts there were, at some stages, few capillaries of the usual size. Instead, there was a maze of enlarged venules and venous sinusoids. These frequently received blood from direct arterial connections (fig. 1). The sinusoids resembled, in a surprising way, those described in the spleen by Kniseley, '36, although they did not have sphincters and it could not be seen that the arteries were sheathed. However, the filtration-filling,

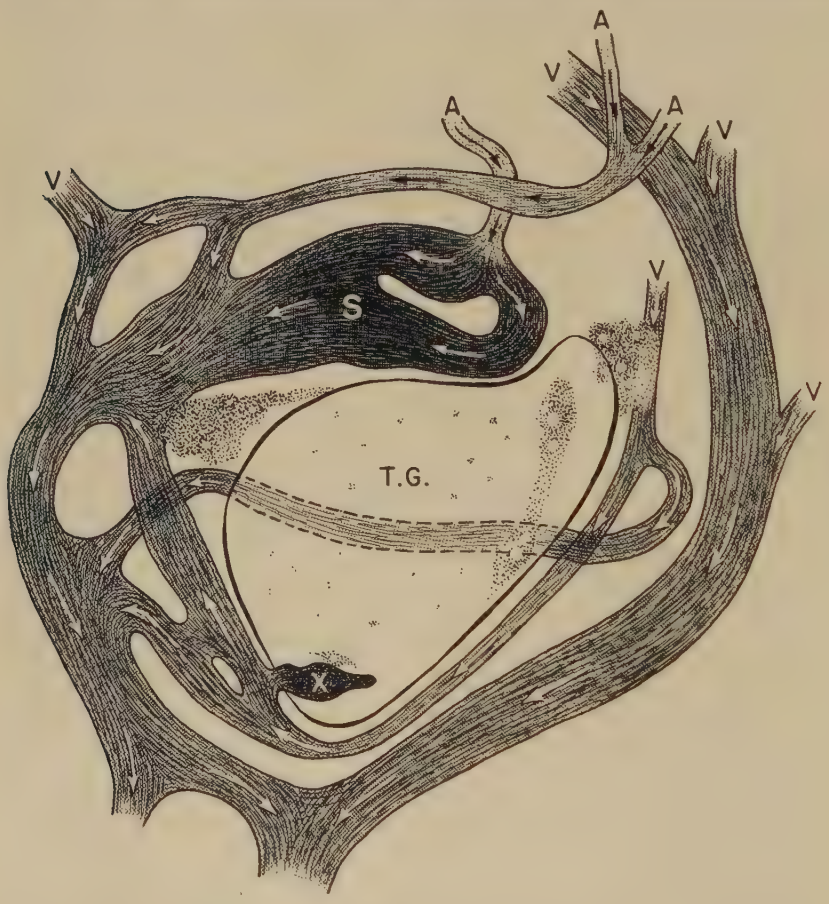


Fig. 1 Camera lucida tracings illustrating a sinusoid, S, near a graft containing both tubule, T.G., and interstitial cells. A refers to artery and V to vein. X = a blood filled vascular sprout. The white islands of connective tissue and endothelium in some vessels eventually disappeared.

storage and emptying (to use the terms of Kniseley) of the blood in the sinusoids was quite similar to that described in the spleen. When a sinusoid emptied into a free flowing vein the thick blood from the sinusoid did not mix immediately with other blood in the vein but moved as a separate column for a considerable distance. This was also observed in the spleen by Kniseley. This is not to imply that there is any relationship between testis and spleen but only to illustrate the nature of much of the circulation in testicular grafts.

The cells in stationary or slow flowing sinusoidal blood formed clumps but no rouleaux. In many sinusoids packed with cells in which no flow occurred for a fairly long period, i.e., one hour, there appeared to have been some plasma loss although it was not extreme. The color of the blood in such vessels was a deep red and individual red cells appeared discrete, unlike the sickly pink color and apparent laking of the red cells which occurs quickly when plasma hemorrhage is acute or the vessel permanently damaged.

Solutions of the dye T 1824 were injected intravenously in one animal on two successive days. The plasma became distinctly blue and the entire skin was blue but no color appeared in the grafts other than in the vessels. There was some sticking of leucocytes to the walls of veins draining graft regions but not in the grafts themselves.

The capacity of venules and sinusoids seemed to be greater than volume of blood entering and leaving them, hence many sinusoids had no flow in them for long periods or the flow might temporarily involve only a portion of the sinusoid. Many instances were seen where very small arteries entered large sinusoids. In these instances red cells piled up in the sinusoid at the mouth of the artery, in much the same manner that sand piles up in a running hour glass, until the sinusoid filled whereupon flow in the artery might stop for a time. If it did not, then the sinusoid might slowly empty. Sinusoids were sometimes slowly emptied of cells without cellular replacement for brief periods. Occasional platelets entering

the sinusoid from the artery indicated that cell free plasma flow produced the emptying.

The vascular characteristics described seemed to be related to the number and activity of interstitial cells. In grafts in which no interstitial cells were visible the blood vessels grew and behaved about as they did in nongraft regions. Around small collections of interstitial cells, 6–10, sufficiently removed from larger aggregations no special vascular response occurred. If the number of cells in small groups increased (the number was then not countable) additional vessels formed among them. If the activity of the cells continued long enough, about 6 weeks, a sinusoidal type of circulation developed. As the vascular bed related to the grafts extended no additional connections with the vessels of the host region were made. This resulted in further slowing of the circulation in grafts.

Although from the beginning of these studies and up to the present, the character of the vascularity in larger grafts was in general as it has been described, there was a wide fluctuation in amount of connective tissue and ground substance, and in the number and size of vessels in individual grafts. This fluctuation occurred periodically and was concommitant with changes in the interstitial cells. During periods when the interstitial cells were increasing in number and when they were becoming most distinct, i.e., when cytoplasmic granules were most numerous and discrete with the centrally placed nucleus discrete, clear and amorphous, then it was that the vessels increased in number and size and sinusoids were most pronounced. This period occupied about 6 weeks. Such a period was followed by one of about two weeks duration in which the reverse took place. Granules in interstitial cells fused and diminished in number, the nucleus became less clear and finally many cells became unrecognizable from the surrounding connective tissue. During this period venous channels and sinusoids diminished in size and number, and the number of vessels of capillary size increased. With the loss of sinusoids the rate of blood flow

in grafts increased. However, the circulation did not lose its predominantly venous character. During a period of regression not all interstitial cells disappeared although they all displayed changes in the cytoplasmic granules.

Interstitial cells at one stage in their history could not readily be confused with any other type of cell although at another stage they might easily be taken for macrophages had one not seen the intervening stages. The appearance and behavior of tubules and interstitial cells will be described in another place.

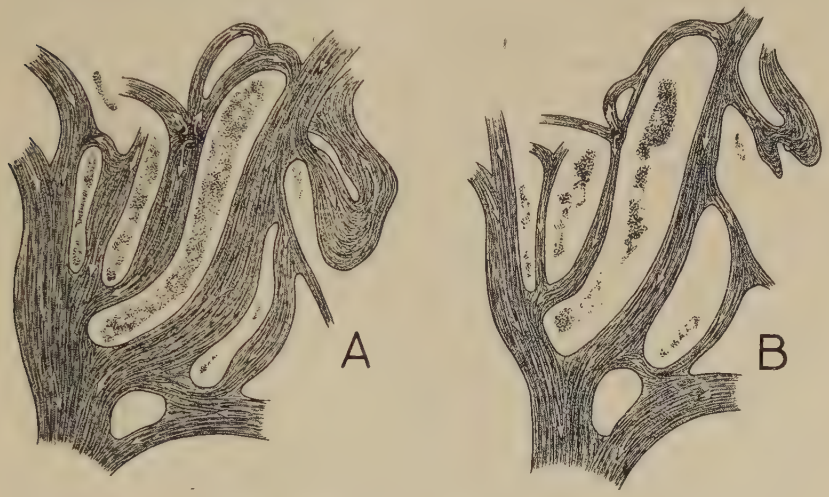


Fig. 2 Camera lucida tracings of the same microscopic field at an interval of 15 days. A indicates the size of interstitial cell masses and of vessels during a period of maximum vascularity. B illustrates the shrinkage of vessels and disappearance of interstitial cells at the beginning of a period of minimum vascularity. No spaces were created in B by the shrinkage of vessels. In A connective tissue and ground substance between vessels are less than in B.

During the periods of maximum vascularity the connective tissue in grafts diminished. This was indicated by the disappearance of connective tissue previously surrounded by sinusoids and by its diminution between enlarging venules (fig. 2). As the vascular extention subsided the connective tissue increased. No spaces were created by the shrinking vascular bed. No data were obtained as to changes in con-

sistency of connective tissue and ground substance. It could have been obtained by removing the cover slip and using micro-dissecting methods. However, such studies require a larger number of preparations than were available.

The periods of maximal and minimal vascularity did not coincide in the two animals that were studied. The time required to reach a maximal from a minimal state, about 6 weeks, was approximately the same in both animals as was the reverse which required about two weeks, but the duration of either phase varied and had no seasonal relationship. Most of the time the vascularity was in a maximal state. Minimal states were observed twice in one animal and once in the other.

In observing the change from minimal to maximal states something was learned about the way sinusoids formed. The first phase involved increase in diameter of capillaries but with no sticking of leucocytes. This would indicate that the internal surface, at least, of the endothelium was undamaged. Dilatation was followed by the slow disappearance of connective tissue between vessels receiving blood from the same source. The endothelium bounding the diminishing connective tissue also disappeared. When that stage was reached in a sufficient number of vessels the capillary bed no longer existed as a conspicuous part of the vascular tree. A sinusoidal circulation supplied largely by direct arteriolar connections was established. All venules increased in diameter. Some vessels acquired aneurysmal swellings which behaved as sinusoids. From these out pouchings new vessels sometimes formed. No changes were observed in vessels with muscle cells on them. The specialized musculature and activity associated with value-like arterio-venous anastomoses was not observed. Regression followed a reverse plan. Venules diminished in diameter and rate of flow in them increased. Some sinusoids with no flow in them slowly diminished in size and disappeared, others regressed to capillary size but a few persisted although reduced in dimensions.

To determine whether or not spreading was influenced by grafts those in the ear opposite the one containing the chamber were utilized. These grafts had been in place for many weeks and could be located as red spots under the skin. India ink, diluted 50% with distilled water, was injected intradermally over some grafts and directly into others. Control injections were made in the tip of the ear beyond the venous drainage of any graft region. Each injection amounted to .05 ml. None of them was massaged. The black areas did not change in size within one hour. However, within 5 hours all areas over grafts had increased, some by as much as 300% whereas the control areas remained unchanged. The areas were measured, under low magnification, daily for 4 days. All areas over grafts increased further but not as much as in the first 5 hours. The control areas remained unchanged.

DISCUSSION

The chief question arising from the foregoing observations is, why was the vascularity in grafts of intersitial cells different from that around grafts of tubules and also different from the vascularity in the normal gland and from that of other rapidly metabolizing tissues as seen in grafts? The possibility that inflammation played a part can be ruled out since once a graft was established there was no leucocytic invasion and no sticking of leucocytes to vessel walls.

Thoma, 1896, suggested that increase in blood pressure and loss of support to vessel walls from changes in surrounding tissue tend to excite new growth of vessels. With the transparent chamber method there is no mechanism in a graft of non-contractile tissue for producing local increase in blood pressure above that which pertains in the chamber as a whole. However, pressure in the vessels of a graft might be lower than in surrounding regions if without change in size of afferent channels enough capillaries and venules enlarged beyond those surrounding the graft. As demonstrated by greater size of vessels and excessive slowing of the blood one may presume that that occurred in interstitial cell grafts and

that pressure in non-arterial vessels in grafts was lower than in comparable vessels outside. Low pressure and stagnation of flow may in themselves be stimuli to vessel formation (Karsner, '42) but, except as alteration in consistency of endothelium may be a factor (Clark, '18), an explanation of the excessive amount and particular type of vascularity in grafts of interstitial cells must be sought outside the vessels themselves.

In most tissues, other than testis, a certain balance is struck between vessel growth and grafted tissue after which little or no change occurs in vascularity. The pattern and behavior of vessels in autogenous grafts of tissue made into an indifferent region, like a vascularized chamber in an ear, tend to duplicate that of the organ from which the graft was obtained. Since in the type of chamber used for these studies nerves and lymph vessels are rarely found, the environment of blood vessels in grafts is regulated by the connective tissue and ground substance of the graft, by the parenchyma of the graft and by the permeability of the vessels. All of these factors are subject to change individually or collectively. If the changes last sufficiently long then they may become manifested in a modified character of the vascularity.

In autogenous grafts of thyroid, adrenal and mammary glands, of lymph nodes, fat, yellow marrow and sympathetic ganglia vascularity reached a certain degree and type and then became stationary. In the case of other tissues studied in this laboratory which, unlike the ones just listed, did not survive for long periods the vascular growth was temporary and as the grafts disappeared the vascular reaction subsided.

Characteristics of the permanent vascular plexuses in interstitial cell grafts which differed from those in other types of grafted tissue were as follows: the plexuses were predominantly venous; there were few capillaries of small size; there were many venous sinusoids and many direct connections between arteries and veins and between arterioles and sinusoids. These were just connections and not the special A.V.A.s. such as those studied by Clark and Clark, '34. In

addition, the development of new vessels extended beyond the borders of grafts and the number and size of vessels fluctuated widely but vascularity in grafts was always much greater than in non-graft regions.

These differences were probably associated with changes in the interstitial cells since they did not occur in grafts of tubules alone. When the interstitial cells underwent changes so did the vascular plexuses. It was difficult to tell which change came first but it is supposed that the interstitial cell changes induced the vascular response probably by producing something which softened the connective tissue and ground substance.

It was demonstrated that testicular grafts produced a spreading factor. Although spreading factors produce change in capillary permeability (Duran-Reynals, '42) as well as in connective tissue, there was little evidence of increased permeability in these studies. Meyer, '47, states that one of the spreading factors, hyaluronidase, when purified produces no change in permeability of vessels. It seems possible that diminution and softening of connective tissue and ground substance preceded development of maximum vascularity thereby diminishing vascular support which acted as a stimulus to vessel growth. The material produced by interstitial cells may also have acted as a direct stimulus to the vessels by changing the character of the endothelium or in some other manner. Clark, '39, states, "it appears that new capillaries will grow in an environment in which the proper physical and chemical conditions are present."

When a graft is vascularized the invading vessels from the host region are accompanied by connective tissue which also fills in the space at the margin of the graft. The connective tissue of the host region presumably contained hyaluronate and hyaluronidase. The graft, it may also be supposed, was high in hyaluronidase since testes contain this enzyme in quantity (Meyer, '47). The graft would, therefore, increase the amount of enzymes in the region and upset the balance between enzyme and substrate thereby inducing alteration in

the connective tissue and ground substance which permitted vessel growth. Such an explanation is only an hypothesis at present.

If the vascularity in interstitial cell grafts was influenced by a spreading factor then it is likely that the factor was produced by the interstitial cells since no unusual vascularity occurred in testicular grafts when those cells were missing. The spreading factor in cryptorchid testes (Duran-Reynals, '42) is much less than in normal testes but is still present. This would indicate that interstitial cells are not the sole source of testicular spreading factors. Any products of interstitial cells which affect the vessels are released by mechanisms which do not impair the cells.

The number of interstitial cells in a graft varied but did not seem to be very great at any time. They could not be counted accurately due to superimposition of cells. But they were certainly fewer than were the parenchyma cells in grafts of thyroid or adrenal of comparable size. In the case of interstitial cell grafts the chief volume of the graft was vascular. One got the impression that in these grafts the vascular plexuses formed in a random manner without regard for the particular vascular requirements of the cells, unless, as there was some evidence to indicate, interstitial cells thrive in a region of very slow circulation.

Mention was made of certain similarities between sinusoids in testicular grafts and those in spleen. This would seem to have no significance except to suggest that given certain conditions outside vessels the vessels tend to conform to those conditions. The vascular pattern in an organ being primarily an expression of conditions in the structures of the organ rather than the result of any inherent ability of vessels to form special patterns irrespective of conditions outside them.

The vessels in interstitial cell grafts resembled those in some types of simple hemangiomas. If the vascularity in such grafts was related, as has been tentatively supposed, to an imbalance between enzyme and substrate, the enzyme

being present in excess, then a similar local imbalance may be a factor in the production of some hemangiomas.

SUMMARY

Autogenous grafts of testis tubules, of interstitial cells and of the two combined survived for months when implanted in tantalum and mica chambers installed in rabbit's ears.

The vascularization of tubule grafts was similar to that observed in cleared specimens of the whole gland injected with India ink. The vascularization of interstitial cell grafts was different. The differences were as follows: the plexuses were predominantly venous; there were few capillaries of small size; there were many venous sinusoids and enlarged venules which received blood by direct arteriolar connection; the plexuses underwent fluctuation from minimal to maximal states but never lost their distinctive characteristics.

Testis grafts produced a spreading factor. Connective tissue and ground substance in interstitial cell grafts changed in amount and probably in character. These changes were concomitant with changes in the interstitial cells and those changes, in turn, coincided with the fluctuation in vascularity.

Consideration was given to the possibility that the unusual vascularity of interstitial cell grafts was related to an imbalance between enzyme and substrate and, since the vessels resembled those in certain hemangiomas, to the possibility that a similar mechanism may be associated with the production of simple hemangiomas.

LITERATURE CITED

Clark, E. R. 1918 Studies on the growth of blood vessels in the tail of the frog larva by observation and experiment on the living animal. Am. J. Anat., *23:* 37.

Clark, E. R., and Eleanor Linton Clark 1934 The new formation of arteriovenous anastomoses in the rabbit's ear. Am. J. Anat., *55:* 407.

———— 1939 Microscopic observations on the growth of blood capillaries in the living mammal. Am. J. Anat., *64:* 251.

Duran-Reynals, F. 1942 Tissue permeability and the spreading factors in infection. Bacteriological Reviews, *6:* 197.

KARSNER, HOWARD T. 1942 Human Pathology, J. B. Lippincott Co., Philadelphia.

KNISELEY, MELVIN H. 1936 Spleen Studies: (1) Microscopic observations of the circulatory system of living unstimulated mammalian spleens. Anat. Rec., *65:* 23.

MEYER, KARL 1947 The biological significance of hyaluronic acid and hyaluronidase. Physiological Reviews, *27:* 335.

THOMA, R. 1896 Textbook of general pathology and pathological anatomy. Translated by Bruce, London.

WILLIAMS, ROY G. 1948 A tantalum chamber for the microscopic study of living cells and tissues transplanted to the rabbit's ear. Anat. Rec., *101:* 291.

PLATE 1

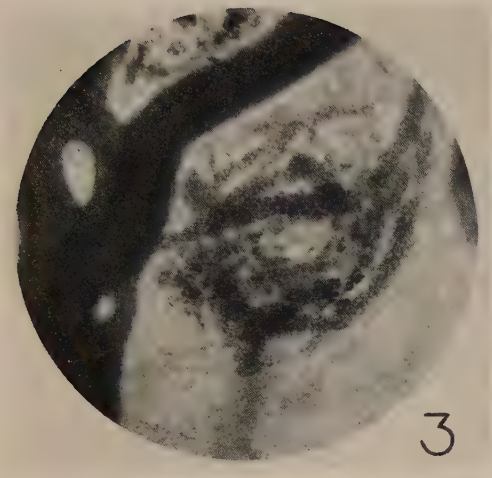
EXPLANATION OF FIGURES

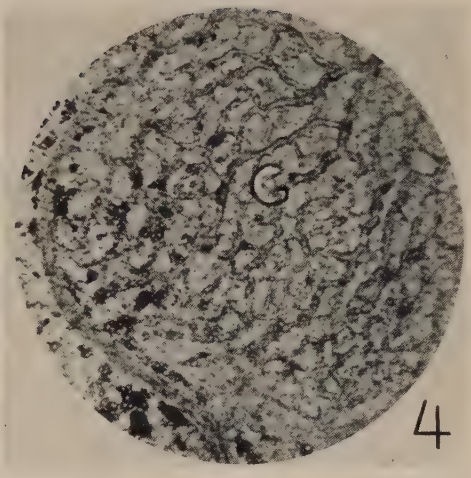
3 Photomicrograph of a small graft of interstitial cells illustrating the venous and sinusoidal nature of the vascularity. Individual interstitial cells are obscured by the vessels.

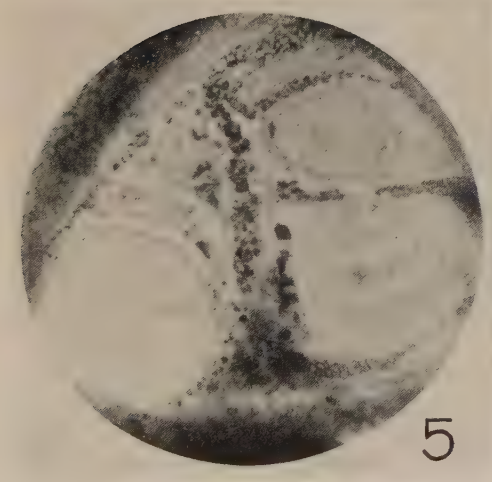
4 Photomicrograph of an autograft from the zona glomerulosa of the adrenal cortex showing the type of capillary plexus commonly seen in gland grafts.

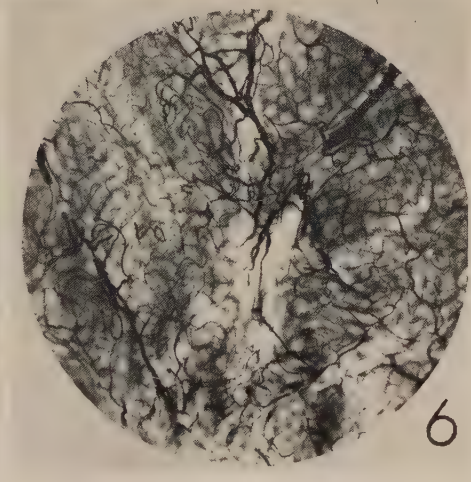
5 Photomicrograph of a tubule graft. The light regions are portions of tubules with vessels forming coarse meshes running over them. Figures 3 and 5 illustrate the differences in vascularity between interstitial cell and tubule grafts.

6 Photomicrograph of a thick cleared section of whole testis in which the vessels had been injected with India ink. It illustrates that the type of vascularity seen in figure 3 does not occur in whole glands or, although it seems unlikely, was not demonstrated in injected specimens.


3


G
4


5


6

CHANGES IN MITOCHONDRIAL FORM[1]

HENRY S. ROBERTS
Department of Zoology, Duke University, Durham, North Carolina

TWENTY-SIX FIGURES

INTRODUCTION

The changes in mitochondria of vertebrate tissues resulting from various experimental methods have been widely studied, and from these studies several distinct schools of thought as to the significance of these changes have developed. By some, of whom Noël ('22) is representative, the changes in form have been interpreted as a functional hypertrophy associated with the elaboration or transformation of certain substances. Other investigators have regarded these changes in form as the mere expression of the physical state of the cytoplasm. For example, Kater ('37) concluded that ". . . the shape and size of mitochondria are entirely dependent on the water content of the tissues . . ." Still others have regarded mitochondria as temporary structures available for the nourishment of the cell. Some investigators have concluded that different mitochondrial forms are expressive of the metabolic activity of the tissues, without regard to the exact role of the mitochondria in the economy of the cell.

Cowdry ('24) has pointed out that three general modes of mitochondrial reaction occur ". . . qualitative, quantitative, and topographical, which may occur singly or in combination . . ." It is significant that the response to so many experimental methods has been observed to be a "granulation," "fragmentation," or "globulation" as it has been

[1] A portion of a thesis submitted in partial fulfillment of requirements of the Ph.D. degree at Duke University.

termed by various authors. The changes described do not appear to be identical, but involve a loss or decrease in number of long filaments and rods and an increase in the number of spherical or rounded structures. Changes in distribution and total number of mitochondria have been described by numerous investigators.

The present study is an attempt to throw further light on the significance of these changes in mitochondrial form by observing the effects of cold, which has been shown to alter mitochondria, on a variety of tissues in the same animal form. Additional observations on mitochondrial changes incidental to a necessary check on the factor of starvation during cold treatment make possible a comparison of the effects of both methods on the same tissues and further to compare the responses of different tissues to the same treatments.

MATERIALS AND METHODS

Larvae of *Ambystoma opacum* were chosen as experimental animals. Since they are poikilotherms the internal temperature can be varied easily, within wide limits, and in a direct relationship with the external temperature. Their small size makes possible isolation and controlled feeding of large numbers of individuals, and offers the added advantage that the entire animal may be sectioned and several tissues studied in the same preparation after identical treatment.

Egg masses were collected, brought into the laboratory and allowed to hatch. As the larvae began feeding they were placed in individual containers. Abnormal and extremely small individuals were discarded. Aquatic oligochaetes, principally *Dero sp.,* constituted the bulk of the diet. This was supplemented by the addition of *Daphnia* or other arthropods of similar size. Sufficient food was placed in each container each day so that an excess remained at the next feeding. At each feeding any fecal pellets were removed as was any uneaten food from the previous day. The water in each container was replaced by fresh spring water every 5th day. The temperature of the laboratory was 22–24°C.,

and the period of light approximately that of daylight during November and December. There were occasional short periods of artificial light at night. Under the above regime growth was quite rapid and development was essentially normal.

For experimental cold treatment individual containers were placed in a cold-drink cooler at 1°C. The cooler was lighted at all times by a 40 watt lamp placed 18 inches from the containers. A temperature of 1° ± 1°C. was maintained in the containers under these conditions. Food was made available, fecal matter removed, and water replaced by spring water at 1°C., as already described. Control animals were maintained under the conditions already described for laboratory rearing. Starved animals were kept under identical conditions except for the lack of food.

After 6 weeks of development under laboratory conditions total body length was recorded for each animal. Measurements of total body length were obtained by the use of a small plastic ruler with the aid of a 10 × magnifier. The measurements are considered accurate to 1 mm. The activity of the animals made more accurate measurement impracticable.

For mitochondrial study the living larvae were dropped into vials of fixative, either Regaud or Champy. The fixative was at 1° C. in the case of cold treated animals and at room temperature otherwise. After initial killing the animals were decapitated and the abdomens opened to facilitate penetration. The material was routinely imbedded in paraffin and sectioned at 6 μ. Acid fuchsin-methyl green stain and iron hematoxylin counterstained with fast green or orange G were used after both fixatives. Most satisfactory results were obtained from Regaud fixation followed by acid fuchsin-methyl green stain.

For a study of liver glycogen as a check on the factor of starvation living larvae were dropped into Lison's fluid at 1°C. in the case of cold treated animals and otherwise at room temperature. After initial killing the animals were de-

capitated and the abdomens opened to facilitate penetration of the fixative. The entire animals were embedded in paraffin, sectioned at 10 μ, and stained with Best's carmine, checked by the saliva test.

Final observations of mitochondria in cold treated animals are based on three animals, 7 days treatment; two animals, 8 days treatment; 5 animals, 10 days treatment; one animal, 22 days treatment; two animals, 24 days cold treatment. Mitochondrial observations on starved animals are based on two animals starved 10 days and two animals starved 22 days. The study on liver glycogen is based on two animals starved 10 days; two animals cold treated 10 days; two animals starved 22 days; two animals cold treated 22 days. In each case double controls were maintained and studied. For each experimental period one normal animal hatched from the same egg mass and reared under identical conditions was sacrificed at the beginning of the period of experimental treatment. A second control animal, also hatched from the same egg mass, was maintained under normal laboratory conditions during the period of experimental treatment and sacrificed at the same time as the experimental animals. In all, 47 animals were sectioned and studied. 24 additional animals were studied under experimental conditions, but no sections were made.

OBSERVATIONS

The effects of cold treatment

I. General

Animals subjected to cold rapidly became extremely quiescent, responding only slightly to pinching of the tail tip with forceps. Some individuals became comatose after varying periods of time and usually died before completion of the experiments. Small animals, less than 35 mm in length, usually withstood the cold better than those of larger size but this was rather variable. No significant changes in size, either increase or decrease, occurred during cold treatment.

No animal was ever observed to feed during the period of cold treatment.

II. Mitochondria

1. General. A study was made of the mitochondria of normal animals ranging in size from 24 to 52 mm in length to determine whether differences existed in the mitochondria of normal animals of different sizes. Liver, pancreas, kidney, intestinal mucosa, gastric mucosa and gastric glands were included in the study. No significant differences within the scope of this problem were found. The same forms and distribution of mitochondria were found in all animals without regard to size. Cell size varied, smaller animals generally having somewhat smaller cells. Figures 1, 2, and 18 show liver mitochondria and cell size of normal animals of different lengths.

As will be shown in the following sections the type of change in mitochondrial form is the same in all animals, although subject to individual variation in degree of change. In general those animals which best withstood the cold treatment showed less marked change in mitochondrial form. However, where carried to the maximum period of 22–24 days all animals, regardless of size, showed the same changes.

On the basis of the above observations all animals of similar treatment were deemed comparable, without regard to original size. Nevertheless, for purpose of study, comparisons were made between experimental and control animals of similar size. Illustrations however have been selected which best demonstrate the point at hand and do not always represent animals of the same size.

A study was made of the following tissues: liver, pancreas, kidney, intestinal mucosa, gastric glands, and gastric mucosa. In no other tissues were the mitochondria preserved and stained in a manner satisfactory for study. The observations on each tissue are given in the following sections.

2. Liver. In the liver cells of the normal *Ambystoma* larvae mitochondria are most frequently seen as rather elongate

filaments. These are shown in figures 1 and 2. The length is occasionally equal to the radius of the cell. The greatest concentrations of mitochondria occur near those surfaces of the cells which border the bile capillaries but some are found in all portions of the cell. Some are always seen in close proximity to the nucleus and frequently appear to be closely applied to the nuclear membrane. In all cells mitochondria of other shapes are to be seen. These include short rods, spherical or oval vesicles, filaments or rods with terminal vesicles, and small granules or spherules. In figures 1, 2, and 18 all of these forms are shown. Although the majority of cells have predominantly filamentous mitochondria as previously described, some cells show fewer filaments and numerous short forms, rods, vesicles, and spherules or granules. Such a condition is shown in figure 18. Although a minority of the cells show the condition illustrated in figure 18, they are a constant feature of the normal liver.

Examination of the cold treated animals reveals a marked progressive change in the condition of the mitochondria. After 8 days granules are quite numerous. Some filaments remain but they are few in number. Short rods are seen and a few vesicular forms. No marked change in distribution is seen in most cells although in some cases a tendency toward concentration around the nucleus is seen. A few of the short rods are quite irregular in form and show a slight tendency toward paler staining. Figures 3 and 4 show liver cells of animals after 10 days cold treatment. Figure 3, from an animal 27 mm long, resembles the condition already described for 8 days cold. Figure 4, from an animal 39 mm long, corresponds closely to that seen after 24 days. It should be noted the difference is one of degree and not of kind. After 24 days cold treatment very few structures other than granules or spherules are seen. There are occasional short rods or irregular club or dumbbell shape and very rarely a vesicle. No definite diminution of mitochondrial substance can be established from this material. Occasionally a reduction

in quantity is suggested but in most cases (fig. 5) there appears to be no change in total amount.

3. Pancreas. The cells of the normal pancreas are usually seen to contain filamentous mitochondria as shown in figures 7 and 19. These are long, strong and seem dense and compact in outline. Short rods and granules, difficult to distinguish clearly from secretion products, occur. Club shapes and dumbbells are rare. In general mitochondria are seen between the nucleus and the proximal cell membrane. The cytoplasm between the nucleus and the gland cavity is ordinarily so crowded with secretion products as to make it difficult or impossible in most cases to determine whether mitochondria are present in this area. In those cells where the secretion products are fewer, some short rods, granules, and vesicular forms are seen. As in the liver, although filamentous forms predominate in most cells of the normal pancreas, in a few cells short rods, granules, and vesicular forms are more numerous.

The changes in mitochondria of the pancreas after cold treatment parallel those already described in the liver. After 7 days cold treatment filaments are few in number. Short rods and granules are most numerous. Vesicular forms are present but rare. The granules present are difficult to distinguish from secretion products but are usually slightly more irregular in outline and are lighter in color. Some short rods are irregular in shape, broad in outline, and stain palely with acid fuchsin. Figure 8 is an animal after 24 days cold treatment. No long filaments or vesicular forms are seen.

4. Kidney. Various regions of the kidney tubule vary greatly in mitochondrial content, and in this material it is not possible to identify positively the various portions. For this reason it is difficult to evaluate the mitochondrial changes occurring during cold treatment, especially from a quantitative point of view. Figures 10 and 20 demonstrate the two extremes of mitochondrial content as seen in the same normal animal. Some tubules are so crowded with strong rods

as to obscure all detail. In other tubules a few fine short filaments and numerous granules, some quite large, are seen. Figure 21 illustrates the condition found after 24 days cold treatment. No tubules contained more mitochondrial material than those illustrated. It is clear then that a transformation of the normal mitochondria to granules or spherules has occurred, and further that definite decrease in the total amount of mitochondrial material, as indicated by the acid fuchsin stain, has taken place. In no other tissues was such a decrease observed. Vesicular mitochondria are seen occasionally in the normal tubule but none have been observed after more than 7 days cold treatment. After 24 days only granules or spherules are seen except for an occasional very short irregular rod.

5. *Intestinal mucosa.* No effort has been made here to analyze carefully the differences which exist between various regions of the intestine since study has shown that the type of change associated with cold treatment is the same for all regions studied. The description and illustrations are of absorptive cells but apply equally well except for distribution to goblet cells. The characteristic mitochondria of the intestinal mucosa may be described as filaments (fig. 12). Some filaments are present immediately around the nucleus and a few scattered ones in all parts of the cell, with a greater concentration beneath the striated border. In the more posterior regions a similar situation exists but the mitochondria are finer, more twisted, more numerous, and the concentrations less marked. Some short rods, granules, and vesicular forms occur in most cells. In some cells of the mucosa filaments are fewer and short rods, granules, and especially vesicles are numerous. This is similar to the situation already described in other tissues. Figure 13 illustrates this condition.

The effect of cold treatment is markedly similar to that already described in the liver, pancreas and kidney. After 24 days cold treatment, except for a rare short irregular rod, all mitochondria are granules or spherules. No rings or

loops have been observed. This condition is well illustrated in figure 14.

6. Gastric glands. Gastric gland cells are not very satisfactory material for the study of mitochondria because of the large quantity of secretion product present and the crowded condition of the cells. In good preparations, however, a color differentiation between zymogen and mitochondria is obtained. Moreover differences in shape and size make differentiation possible. Certainly it is possible to differentiate at all times between mitochondrial filaments, rods, oval vesicles, and zymogen "granules." The latter are more easily confused with granular mitochondria, but the larger size, more perfectly spherical outline, and stain differentiation of the zymogen makes identification fairly certain. In normal cells the secretion products, zymogen "granules," are thickly packed into the apices of the cells with some scattered material in the vicinity of the nuclei. Mitochondria are usually observed in the proximal half of the cell but are probably present in other regions though obscured by secretion products. In figure 22 one cell has been sectioned so that the nucleus does not show. Here the characteristic long filaments are seen. Short rods, vesicles, and granules are also present.

The effect of cold treatment on mitochondria of the gastric gland cells is similar to that already seen in the other tissues studied but is more difficult to observe and analyze. Figure 15 shows a single gland from an animal after 24 days cold treatment. At first glance it appears similar to those of the normal animal but closer observation reveals distinct differences. The apices of the cells are vacant whereas in the normal animal they are filled with zymogen. Numerous granules are present in the cells but they are concentrated about the nuclei on all sides, a distribution not characteristic of zymogen granules. Zymogen, where present in this preparation, is stained black in color but the granules illustrated are pink. On the basis of distribution, stain differentiation, and slight differences of size and shape, they are identified

as granular mitochondria. Study of intermediate stages of 7 and 10 days reveals conditions intermediate between normal animals and those subjected to 24 days cold treatment.

7. Gastric mucosa. The gastric mucosa cells of the normal larvae are similar to those of the transitional region of the intestine already described. Figures 16 and 23 illustrate the observed condition. Three areas of concentration exist, just below the mucous cap, at the base of the cell and around the nucleus. Some mitochondria are present in all regions of the cell. Long filaments predominate in most cells but other forms, short rods, loops, rings, and granules are also present. Some cells show relatively few filaments and an increased number of rods, vesicular forms, and granules.

The changes occurring after cold treatment follow the pattern already observed. After 24 days almost no forms other than granules are found (fig. 17). Very rarely a short rod is seen. Vesicular forms are absent or rare. Some tendency toward clumping of the granular mitochondria is seen but there are no marked changes in distribution.

III. The factor of starvation

It has already been noted that no animal was ever observed to feed during the period of cold treatment. Consequently the degree, if any, to which starvation may have affected the results described is of importance. Those animals which were starved showed a progressive emaciation and decreased pigmentation. After 22 days the trunk was exceedingly slender and rather angular in outline. The color was appreciably lighter than that of normal animals. During the period of starvation they remained very active, even after 22 days. Animals which were cold treated showed no such emaciation or changes in pigmentation. They were inactive during the entire cold treatment, not moving except on strong stimulation. Cold treated animals showed an average decrease in length of .64 mm after 10 days, based on 21 animals, and of .75 mm after 22 days, based on 10 animals.

Since the method of measurement was accurate only to 1 mm the decrease is not regarded as significant. Starved animals showed an average decrease in length of 3.08 mm after 10 days, based on 6 animals and of 5.83 mm after 22 days, based on 6 animals. The control animals showed an average increase in length of 4.7 mm after 10 days, based on 5 animals, and of 8.6 mm after 22 days, based on 6 animals.

The glycogen study made revealed marked differences between starved and cold treated animals. Figures 24, 25, and 26 illustrate differences in amount of liver glycogen in normal, starved, and cold treated animals. The quantity of glycogen present in cold treated animals is substantially the same as that in normal animals. Animals starved a corresponding length of time contain practically no liver glycogen.

Certain other differences are demonstrated by the same material. The differences in cell size are striking. The cells of starved animals are quite small as compared with those of normal animals. Some slight decrease in size is suggested in the cold treated animals also, but this is by no means so marked as in the starved specimens. The intercellular spaces in starved animals are much larger than those seen in normal and cold treated ones. When the abdomens were opened at the time of fixation the livers of starved animals were seen to be greatly reduced in size.

The effects of starvation

The period of starvation in these tests was not extended beyond 22 days. The effects of starvation on mitochondrial form differed in degree from those resulting from cold, but the type of change observed was essentially similar. For this reason detailed account of the mitochondrial changes observed is not contemplated.

Hepatic mitochondria of starved animals show a greater degree of change than do those of other tissues studied. After 22 days the mitochondria are predominantly granular or spherical, but short rods are fairly numerous and there is no clumping of mitochondria around the nuclei such as is

found after corresponding periods of cold treatment. This condition is shown in figure 6. A comparison of this figure with the condition existing after 24 days cold treatment (fig. 5) will demonstrate that the type of change occurring is essentially the same.

Mitochondria of the other tissues studied show less change after starvation, but here too the difference is one of degree, and not in type of change. In the pancreas and kidney the mitochondria are primarily filamentous, but short rods and granules are more numerous than in the normal animal. Vesicular mitochondria, absent after prolonged cold, are fairly numerous. These tissues are illustrated in figures 9 and 11.

IV. Growth and development

The degree of growth and development during experimental treatment is important as an indicator of the effect of the treatment on biological processes. In this work no attempt has been made to determine precisely the degree of differentiation of various organs and systems. Instead more general indicators of development have been used.

One of these, change in size, has already been discussed in connection with the factor of starvation. It was shown that no significant changes in total body length, either increase or decrease, occurred during cold treatment. Significant decreases in length were found after starvation and significant increases after normal growth, during corresponding periods of time.

Nuclei appeared essentially normal in all animals. Mitotic figures, however, showed significant differences. Mitotic figures were absent or exceedingly rare in starved animals, a phenomenon that might be expected. In normal animals all stages of mitosis were found in relative abundance. Cold treated animals showed numerous mitotic figures, as many or more than the normal animals, but no phases later than the metaphase were observed.

DISCUSSION

I. Method

The rate of biological processes generally increases on heating and decreases on cooling within the limits of the biokinetic temperature When the lower limit of the biokinetic range is reached biological processes are arrested. This condition is reversible if not too prolonged. It may occur at temperatures above or below 0°C. according to the sensitiveness of the animal (Bělěhrádek, '35). The temperature used in this series of experiments, 1° ± 1°C. was selected as it is above the freezing point but below the minimum temperature normally encountered by the experimental animals in nature. That this temperature is, or approaches closely, the lower limit of the biokinetic range is indicated by the death rate after long exposure and by the comatose condition of the animals undergoing treatment. Thus the normal metabolism of the experimental animals was greatly altered by the method used.

It seems quite clear that cold and not starvation is responsible for the mitochondrial changes observed. Even though the animals did not feed during the period of treatment, no evidences of starvation were observed. McCurdy ('39) showed that in the livers of starved salamanders the degree of mitochondrial change was proportional to the depletion of stored glycogen and fat. It has been demonstrated here that after cold treatment, the amount of stored glycogen approximated that of normal animals, but after corresponding periods of starvation was almost entirely absent. Mitochondrial changes after 22 days starvation approximated those of 7 days cold treatment.

II. Significance of mitochondrial transformation

It has been demonstrated that the changes in mitochondrial form resulting from cold treatment are essentially similar in all glandular tissues studied: liver, pancreas, kidney, gastric glands, gastric epithelium, and intestinal epithelium. In all

of these tissues the longer forms predominate in normal animals but short rods, vesicular forms, and granules are a constant feature. During cold treatment there is a progressive decrease in the number of long rods and filaments and an increase in the number of granules and spherules. Vesicular forms almost disappear during cold treatment. Further it has been shown that the changes resulting from starvation, though differing in degree during corresponding periods of time, are essentially similar to those resulting from cold treatment. A careful study of the literature and of accompanying illustrations suggests very strongly that changes of mitochondrial form, though not identical, are essentially similar, regardless of the type of experimental treatment employed.

The transformation of filamentous mitochondria to granules or spherules in various tissues after exposure to heat or cold has been described by numerous investigators, among them Meola ('34: heat — guinea pig pancreas); Bailiff ('37: lethal heat and cold — rat thyroid); MacCardle ('37: heat and cold — liver of *Fundulus* and goldfish); and Kater ('37: cold — rat liver).

The effects of starvation, resulting in a loss of filamentous mitochondria and increased number of granules, have been investigated by Miller ('22: stomach, duodenum, and pancreas of the rat); Okuneff ('23: dog liver, kidney, and pancreas); Ma ('24: guinea pig pancreas); Hall and Mackay ('33: rabbit liver); McCurdy ('39: liver of salamanders); and Williams ('43: salamander intestinal epithelium).

Chemical agents resulting in granulation of mitochondria have been investigated by Scott ('16: phosphorous poisoning — pancreas of white mice); Nicholson ('24: injection of potassium iodide, atropine sulfate, pilocarpine, morphine sulfate, potassium arsenite, small doses of hydrogen cyanide, phosphorus, inhalation of pure oxygen, carbon dioxide, and hydrogen — thyroid of guinea pig and rabbit); Johnson ('31: cyanide — frog kidney); Clark and Hair ('32: etherization —

frog kidney); Kater ('31: etherization — cat liver); and Turchini and Meites ('43: chloroform).

Vitamin free diets which resulted in mitochondrial granulation have been described by Miller ('22: vitamin A — gastric gland cells, duodenum, and pancreas of rat); Nicholson ('24: water soluble vitamin B — thyroid of rabbit and guinea pig); and Bourne ('35: vitamin C — adrenal cortex, liver, kidney, and ovary of the guinea pig).

Endocrine imbalance has been shown to result in the transformation of filamentous mitochondria to granules by Steffens ('41: hypophysectomy — rat liver); Cramer and Ludford ('26: thyroid feeding — rat thyroid); Nicholson ('24: thyroxin and adrenalin — guinea pig thyroid; Clark and Hair ('32: adrenalin — frog liver); Kater ('31: adrenalin and insulin — liver of cat; '37: adrenalin — liver of rabbit but no effect on guinea pig and rat); and DeRobertis ('40: parathyroid extract — rat parathyroid).

Noël ('22), confirmed by Noël and Pallot ('33) and Steffens ('41) has shown that the feeding of egg white to rats resulted in an enspherulation of hepatic mitochondria.

Nicholson ('24) working with the guinea pig liver and Emmel ('40) with the rat kidney found a transformation of mitochondria to granules following occlusion of the arterial blood supply.

Certain results which are inconclusive or apparently contradictory to those which have been listed must be mentioned. Kater ('37) subjected white rats to a temperature of 45°C. for a period of one hour and found the mitochondria to be slender filaments. These results, so different from those obtained by other investigators, can probably be explained on the basis of the short time of exposure and the homoiothermic nature of the animals used. Internal temperatures were not checked. Nicholson ('24) found the injection of insulin to have no effect on thyroid mitochondria of the guinea pig and Kater ('37) found the injection of adrenalin to have no effect on the hepatic mitochondria of guinea pig and rat.

The series of papers listed here is by no means a complete review of the literature. Only a few of the more representative papers have been cited, but they are typical of the work done and results obtained. In view of of the varied techniques and animals which have been employed it seems futile to attempt to evaluate the changes observed after cold treatment and starvation in terms of the particular experimental treatment used. Rather it is necessary to interpret them as illustrative of the effects of a wide variety of experimental treatments.

Guillermond ('34) has rviewed the evidence supporting the view that these changes in form are a "functional hypertrophy" resulting from a secretion or transformation of certain substances, and reached the conclusion that only the work of Noël ('22), later confirmed by Noël and Pallot ('33) and by Steffens ('41), can be regarded as definitely supporting such a view. Serious theoretical objection can be raised to the interpretation made by Noël and Noël and Pallot. In each of the papers referred to a pure protein diet, consisting of egg white, was found to result in a marked enspherulation of hepatic mitochondria. However, the feeding of raw or insufficiently cooked egg white is known to produce characteristic toxic symptoms and in a short time death. The literature on this subject is adequately reviewed by Parsons and Kelley ('33) and du Vigneaud ('42). Raw egg white has been shown to contain a protein substance, avidin, which decreases the biotin of the body, thus causing certain pellagra-like symptoms and death. Biotin is stored primarily in the liver and kidney. The results obtained by Noël, Noël and Pallot, and Steffens can probably be ascribed to this factor and not to any direct correlation between protein metabolism and mitochondrial form. Although this theoretical objection is quite strong and convincing, it should be confirmed by laboratory investigation. No evidence has been found in the present work to support the theory of functional hypertrophy. If we examine this interpretation in the light of generally

similar changes resulting from quite different methods it becomes still less convincing.

The view of Kater ('37), that changes in mitochondrial form are the result of changes in the physical state of the cytoplasm, in this case water content, has not been generally accepted. The method of water determination used by Kater, dehydration of the liver until no further decrease in weight occurred, makes no distinction between intracellular and extracellular water. McCurdy ('39) showed that it was virtually impossible to predict the form of mitochondria on the basis of the water content of the tissue. Further it is impossible to show that the varied experimental methods which have been demonstrated to result in an essentially similar change of form are similar in their effect on the water content of the cell. The related view of Clark and Hair ('32) that change of mitochondrial form is associated with alterations in the physical state of the ground cytoplasm, as expressed in the glucose-glycogen equilibrium, cannot be supported for the same reason. It also seems clear that viscosity changes of the cytoplasm are not responsible for changes of mitochondrial form. It is generally agreed (Beléhrádek, '35; Heilbrunn, '28, '43) that either increase or decrease of temperature, beyond the optimum range, results in increased viscosity. Chloroform (Turchini and Meites, '43) and ether (Clark and Hair, '32; Kater '31) in anaesthetic concentrations, which have been shown to produce mitochondrial changes similar to those resulting from heat and cold, result in a decreased viscosity of cytoplasm.

Mitochondria have been regarded as reserve material for cellular metabolism. Guthrie ('25) regarded mitochondria as a complex of phospholipins and protein precipitated out of the saturated cytoplasm. More recently Bensley ('47) has suggested that mitochondria are temporary aggregates of complex composition which may be utilized for the nourishment of the cell. The disappearance of mitochondria from pancreatic cells aftr prolonged inanition is interpreted as the result of the utilization of mitochondria for the nourish-

ment of the cells; the present work does not support this view. A marked decrease in the mitochondrial material of kidney cells occurred after cold treatment even though there was no evidence of starvation. In animals starved for 22 days and showing marked evidences of inanition, such as decrease in length and shrunken cells, there was no appreciable diminution in the amount of mitochondrial material in any of the tissues studied.

The basic similarity of the changes of mitochondrial form resulting from various experimental methods has been emphasized. The various theories of the significance of this change of form which have been discussed are all subject to the same objection. They are attempts to interpret this phenomenon in the light of the particular technique or techniques employed and fail to consider the essentiallly similar results which have been obtained by quite different methods. The one effect on the organism which is shared by all of these methods is that each affects the normal metabolism of the cell. It does not seem possible to demonstrate any other effect common to all. Considered from this point of view the granulation of mitochondria in response to various experimental methods can be interpreted as an expression of the alteration of the normal cellular metabolism. The excellent work done recently on the isolation of mitochondria by centrifugation has already yielded much information, especially as to enzyme localization within mitochondria. It seems probable that it is from work of this type coupled with cytochemical techniques that added knowledge of mitochondria is to be obtained, rather than from a study of mitochondrial change of form.

SUMMARY AND CONCLUSIONS

Larvae of *Ambystoma opacum* were subjected to a temperature of 1° ± 1°C. for periods ranging from 7 to 24 days. As a check on the factor of starvation other animals were maintained under normal laboratory condtions except that food was withheld. Controls were maintained and studied for each period of the experimental treatment.

A comparison of the mitochondria of normal and cold treated larvae showed that in the liver, kidney, pancreas, gastric glands, gastric mucosa, and intestinal mucosa, there was, during exposure to cold, a decrease in the number of long filamentous mitochondria and an increase in the number of shorts forms, especially granules and spherules. After 24 days long forms were entirely absent. Vesicular mitochondria, numerous in the normal animal, were absent after prolonged exposure.

Histochemical tests for stored liver glycogen and a study of size changes showed the results described to be the effect of cold and not caused by starvation resulting from failure of the experimental animals to eat during cold treatment. Mitochondrial changes in animals starved for 22 days were similar to those resulting from 7 days cold treatment.

The change of mitochondrial form observed is believed to be essentially similar to that resulting from a wide variety of experimental techniques employed by other investigators and not a unique response to the methods employed.

It is concluded that change of mitochondrial form is an expression of an alteration of normal cellular metabolism and further that it is not possible to reach valid conclusions as to the role of mitochondria in vital processes on the basis of the change of form resulting from a particular experimental technique.

ACKNOWLEDGMENT

I wish to express my appreciation to Dr. G. T. Hargitt for his suggestions and criticism throughout this problem, and to Mr. R. L. Rigsbee for his aid in preparation of the illustrations.

LITERATURE CITED

BAILIFF, R. N. 1937 Cytological changes in the rat thyroid following exposure to heat and cold, and their relationship to the physiology of secretion. Amer. Jour. Anat., *61:* 1–18.

BELĚHRÁDEK, J. 1935 Temperature and living matter. Protoplasma Monographien, Bd. 8. Gebruder Borntraeger, Berlin., x + 277 pp.

BENSLEY, R. R. 1947 On the nature of the pigment of mitochondria and of submicroscopic particles in the hepatic cell of the guinea pig. Anat. Rec., *98:* 609–619.

BOURNE, G. 1935 Mitochondria, Golgi apparatus, and vitamins. Australian Jour. Exp. Biol. and Med. Sci., *13:* 239–249.

CLARK, J. A. AND G. E. HAIR 1932 Observations on the mitochondria of the hepatic cell of the frog in normal and hyperglycemic state. Zeitschr. f. Zellforsch. u. mikr. Anat., *15:* 123–130.

COWDRY, E. V. 1918 The mitochondrial constituents of protoplasm. Contrib. Embryol. Carnegie Inst. Washington. *8:* 39–160.

———— 1924 General cytology. Ed. E. V. Cowdry. pp. 311–382. University of Chicago Press, Chicago, Ill. viii + 754 pp.

CRAMER, W. AND R. J. LUDFORD 1926 On cellular activity and cellular structure as studied in the thyroid gland. Jour. Physiol., *61:* 398–408.

DEROBERTIS, EDUARDO D. P. 1940 The cytology of the parathyroid gland of rats injected with parathyroid extract. Anat. Rec., *78:* 473–495.

EMMEL, V. M. 1940 Mitochondrial and pH changes in the rat's kidney following interruption and restoration of the renal circulation. Anat. Rec., *78:* 361–381.

GUILLERMOND, A. 1934 Les constituants morphologiques du cytoplasme: le chondriome. Actualités Scientifiques et Industrielles, No. 170. Herman and Cie. Paris 128 pp.

GUTHRIE, M. J. 1925 Cytoplasmic inclusions in cross-activated eggs of teleosts. Zeitschr. f. Zellforsch. u. mikr. Anat., *2:* 347–381.

HALL, E. M. AND E. M. MACKAY 1933 The relation between the mitochondria and glucose-glycogen equilibrium in the liver. Amer. Jour. Pathol., *9:* 205–220.

HEILBRUNN, L. V. 1928 The colloid chemistry of protoplasm. Protoplasma Monographien. Bd. 1. Gebruder Borntraeger, Berlin. vii + 356 pp.

———— 1943 An outline of general physiology, 2nd ed. W. B. Saunders Co., Philadelphia. xii + 748 pp.

JOHNSON, J. L. 1931 The effects of cyanide on the batonettes of the frog's kidney. Anat. Rec. *51:* 71.

KATER, J. McA. 1931 Variations in the mitochondria of the hepatic cell in relation to alterations of the glycogen-glucose equilibrium. Anat. Rec., *49:* 277–290.

———— 1937 The liver-blood fluid exchange and the morphology of hepatic cell mitochondria. Jour. Morph. *61:* 473–484.

MCCURDY, M. B. D. 1939 Mitochondria in liver cells of fed and starved salamanders. Jour. Morph., *64:* 9–35.

MA, W. C. 1924 The changes in the pancreatic cell of the guinea pig during inanition and refeeding. Anat. Rec., *27:* 47–60.

MACCARDLE, R. C. 1937 The effects of temperature on mitochondria in liver cells of fish. Jour. Morph. *61:* 613–639.

MEOLA, F. 1934 The effect of elevated temperature on the mitochondria of the guinea pig pancreas. Anat. Rec., *60:* 5–7.

MILLER, S. P. 1922 Effects of various types of inanition upon the mitochondria in the gastro-intestinal epithelium and in the pancreas of the albino rat. Anat. Rec., *23:* 205–210.

NICHOLSON, F. M. 1924 An experimental study of mitochondrial changes in the thyroid gland. Jour. Exp. Med. *39:* 63–75.

NOËL, R. 1922 Recherches histo-physiologiques sur la cellule hépatiques des mammiferes. Arch. Anat. Micr., *19:* 1–158.

NOËL, R. AND G. PALLOT 1933 Recherches sur le chondriome de la cellule hépatique des mammiferes. I. Le chondriome hépatique chez la rat. Bull. Histol. App. Physiol. et Pathol. *10:* 169–186.

OKUNEFF, N. 1923 Studien über Zellveränderungen in Hungerzustände. (Das Chondriom.) Arch. f. mikr. Anat., *97:* 187–203.

PARSONS, H. T. AND E. KELLEY 1933 The effects of heating egg white on certain characteristic pellagra-like manifestations produced in rats by its dietary use. Amer. Jour. Physiol., *104:* 150–164.

SCOTT, W. J. M. 1916 Experimental mitochondndrial changes in the pancreas in phosphorous poisoning. Amer. Jour. Anat., *20:* 237–253.

STEFFENS, H. W. 1941 An experimental study of the mitochondria in the hepatic cells of the white rat. Anat. Rec., *81:* 243–263.

TURCHINI, M. AND M. MEITES 1943 Modifications subies par le chondriome hepatique au cours de l'anesthesie chloroformique. Bull. Acad. Sci. et Lettr. Montpelier., *73.*[2]

———— 1946 Ibid. Biol. Abstr. 20 (*10*): 2042, item 19282.

DU VIGNEAUD, V. 1942 The biological action of the vitamins, pp. 144–168. Ed. by E. A. Evans, Jr. Univ. of Chicago Press, Chicago, Ill.

WILLIAMS, M. A. 1943 Mitochondria in the intestinal epithelial cells of starved and fed salamanders. Anat. Rec., *85:* 195–209.

[2] Read in abstract only.

PLATE 1

EXPLANATION OF FIGURES

Figures 1–17 were drawn with the aid of a camera lucida, using oil immersion objective and 10 × ocular. The magnification as reproduced is approximately × 900. All figures are from sections stained with acid fuchsin and methyl green after Regaud fixation unless otherwise noted.

1 Hepatic cell of control animal, length 39 mm, showing characteristic filamentous mitochondria, granules, and vesicular forms.

2 Hepatic mitochondria control animal, length 24 mm.

3 Hepatic mitochondria of 27 mm animal after 10 days cold treatment.

4 Hepatic mitochondria of 39 mm animal after 10 days cold treatment.

5 Hepatic mitochondria of 20 mm animal after 24 days cold treatment.

6 Hepatic mitochondria of 36 mm animal after 22 days starvation. Compare with figures 4 and 5.

7 Pancreatic mitochondria of 45.5 mm control animal. Zymogen granules are stippled.

8 Pancreatic mitochondria of 32 mm animal after 24 days cold treatment. Zymogen granules are stippled.

9 Pancreatic mitochondria of 36 mm animal after 22 days starvation.

10 Kidney mitochondria of 41 mm control showing maximum and minimum concentrations in adjacent tubules.

11 Kidney mitochondria of 36 mm animal after 22 days starvation, showing slight change of form.

12 Intestinal mucosa of 39 mm control showing predominantly filamentous mitochondria.

13 Intestinal mucosa, same slide and section as figure 12, showing numerous vesicular forms.

14 Intestinal mucosa of 32 mm animal after 24 days cold treatment. Note absence of filaments and vesicles.

15 Gastric gland cells of 32 mm animal after 24 days starvation. Zymogen is absent. Note vacant apices of cells. Granular mitochondria with a few short rods are seen.

16 Cells of gastric mucosa, 38 mm control, showing characteristic mitochondria.

17 Cells of gastric mucosa of 20 mm animal after 24 days cold treatment. Other than granules only an occasional short rod is seen.

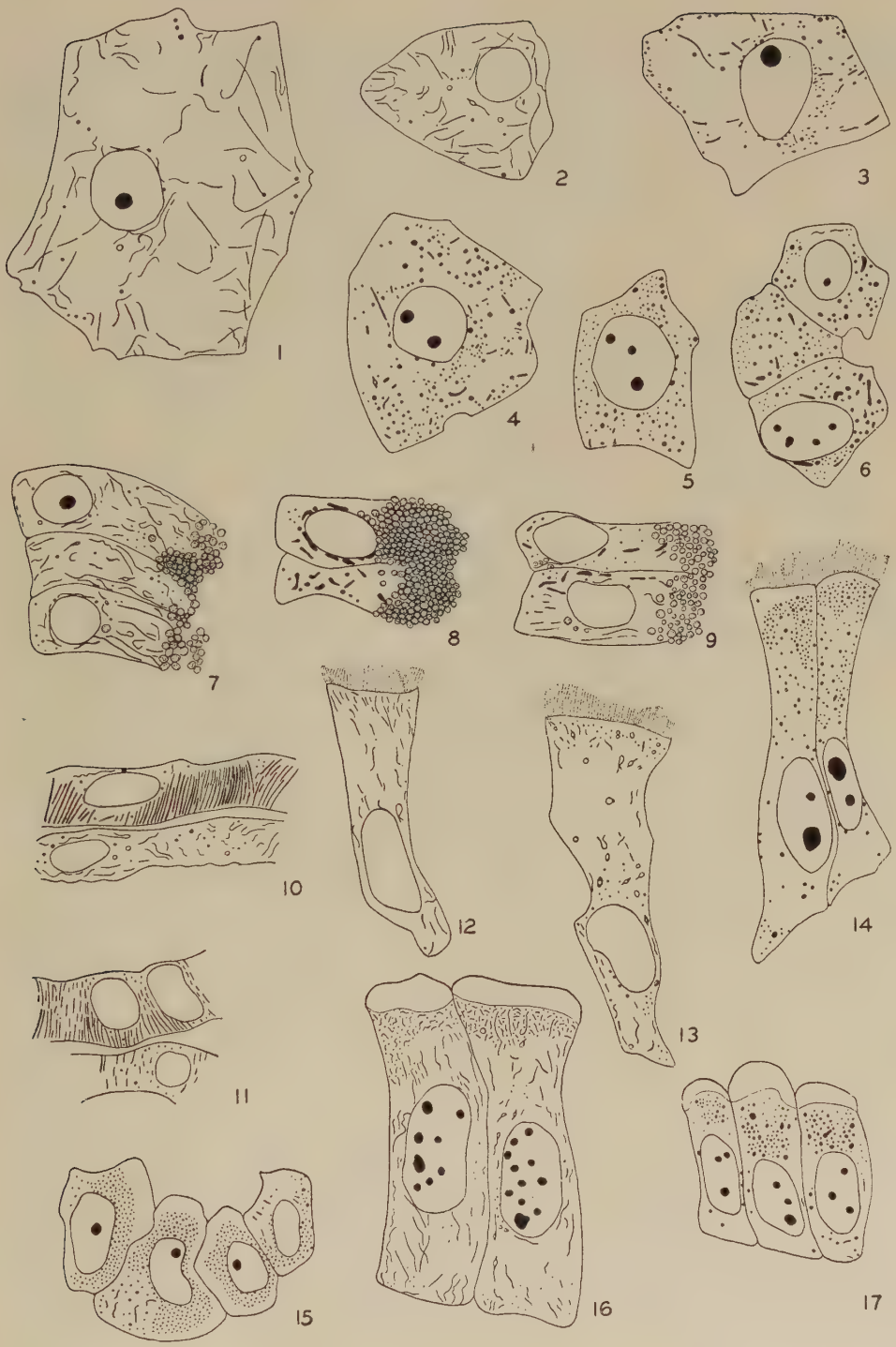

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17

PLATE 2

EXPLANATION OF FIGURES

Figures 18–26 are unretouched photomicrographs. Figures 18–23 were made with oil immersion objective and 10 × ocular and are reproduced at a magnification of approximately × 950. Figures 24–26 were made with high-dry objective and 10 × ocular and are reproduced at approximately × 415.

18 Hepatic cells of 39 mm control showing numerous vesicular mitochondria.

19 Pancreatic mitochondria, same animal as figure 7.

20 Kidney mitochondria of control animal. Compare with figure 10.

21 Kidney mitochondria of 32 mm animal after 24 days cold treatment. Note absence of forms other than granules except for a few short rods.

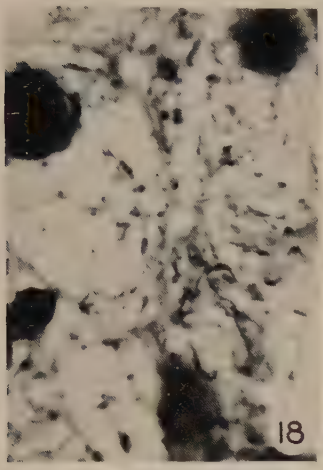
22 Gastric gland mitochondria of 37 mm control. Zymogen granules appear as dense black spheres.

23 Gastric mucosa of 37 mm control showing characteristic long mitochondrial filaments.

24 Liver glycogen of 49.5 mm control. Lison fixation, Best's carmine stain. Glycogen appears as dark irregular plaques in the photograph.

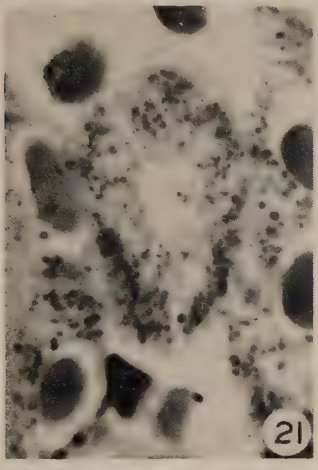
25 Liver cells of 33.5 mm animal after 22 days starvation. Identical stain and fixation as in figure 24. Glycogen almost totally absent. Note the shrunken cytoplasm. Prolonged photographic exposure was required to register the almost transparent cytoplasm.

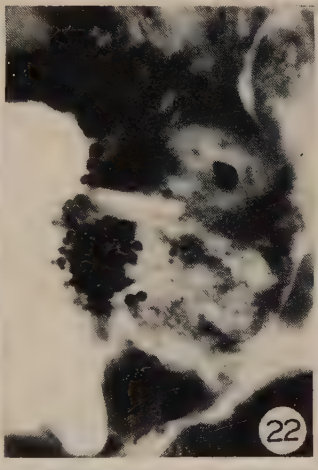
26 Liver glycogen of 37.5 mm animal after 22 days cold treatment. Fixation and stain identical with that in figures 23 and 24. Glycogen is as abundant as in the normal animal.

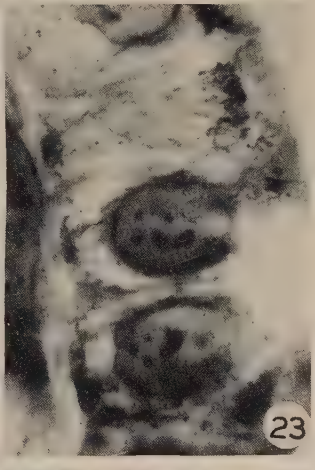

18

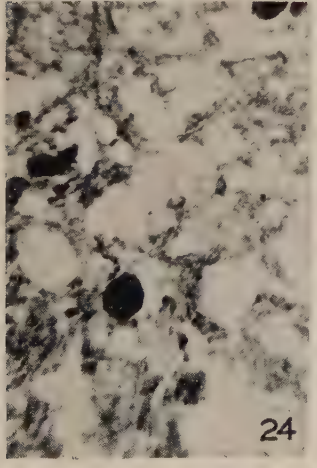
19

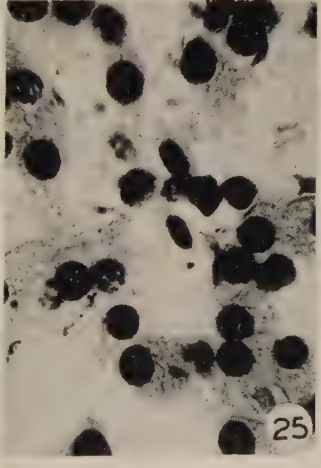
20

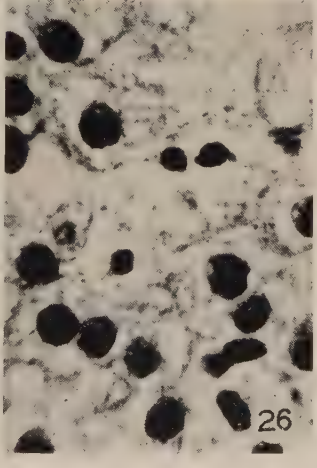

21


22


23


24


25


26

THE EFFECT OF ALIMENTARY FACTORS ON LIVER GLYCOGEN RHYTHM AND THE DISTRIBUTION OF GLYCOGEN IN THE LIVER LOBULE

CARL-AXEL EKMAN AND HJALMAR HOLMGREN
The Department of Experimental Histology, Karolinska Institutet, Stockholm, Sweden

EIGHT FIGURES

During the last 20 years the cyclic function of the liver (Forsgren, '27, '35; Holmgren, '36; Jores, '35; Nordenskiöld, Sjögren, Holmgren and Möllerström, '38; Ågren, Wilander and Jorpes, '31) characterized by regularly recurring phases of glycogen storage and depletion has been the subject of lively discussion. It has been maintained that the rhythmic work of the liver is to a large extent independent of feeding. A view which differs so widely from earlier ideas, has, of course, been subjected to criticism (Higgins and co-workers, '32, '33; Deane, '44). These critics maintain that glycogen variations are entirely due to alimentary factors and that there is in fact no cyclic liver function.

Higgins et al. ('32, '33) have investigated the dependence or independence of the cyclic function of the liver on alimentary factors in experiments with animals which have been fed at definite times of day for long or short periods and then killed the following day. In view of these authors, the results show that the cyclic function is wholly conditioned by alimentary factors.

It may be objected that these experiments constitute a serious infringement of the habits of the animal, likely to conceal any normal regular variations. Experiments which include only one period of feeding over a period of two or three hours must involve a combination of full feeding and

starving. It has been demonstrated with mice (Swensson, '45) that after only 5 or 6 hours starvation liver glycogen begins to sink.

Forsgren ('35), Nordenskiöld et al. ('38) and Ågren et al. ('31), on the other hand, have demonstrated a liver glycogen rhythm during fasting for 24 to 48 hours. They maintain that a cyclic function is to be found in animals such as rabbits which always have food in their stomachs. These results show that the cyclic liver function is not, or is only secondarily, dependent on alimentary factors.

To these experiments it may be objected that when the supply of food has not been regulated, the feeding habits of the animal may determine a rhythm in the content of glycogen in the liver and the variations would then be dependent on alimentary factors. This objection is answered by experiments with an animal like the rabbit, which always has food in its alimentary tract. When the mealtimes of the rat are distributed with short intervals over practically the whole 24 hours (Holmgren, '36), a steady supply of food must be assumed. Finally, glycogen shows clear cyclic day and night variation both after 24 hours and 48 hours fasting (Ekman and Holmgren, '47; Nordenskiöld et al., '38; Ågren et al., '31).

It is obvious that glycogen variation in the liver is dependent on a supply of raw materials. The question is whether an animal with free access to food eats in response to external or internal factors. If the latter is true, the cyclic principle can hardly be denied. It is of interest, moreover, that absorption of fat in the intestines is cyclic (Holmgren, '36) and that the absorption of glucose varies through the 24 hours (Hamar, '41).

This study takes up the following questions:

1. Does cyclic function of the liver occur when the stomach contains food during the day and during the night?

2. Is there, in the liver lobule, a definite pattern of glycogen deposit which corresponds to the various functional phases of the organ?

MATERIAL AND METHODS

Male white mice (195 animals) and white rats of the same stocks were used. Corresponding experiments were carried out at the same time of the year to avoid seasonal and temperature variations (Swensson, '45). The experiments have been carried out in 4 stages, three of these with mice.

Before the experiments, the mice have fasted for 24 hours and then have had free access to food consisting of: white bread, 100 gm, sugar, 25 gm and vitamin mixture (Viterna), $\frac{1}{2}$ cm^3. Animals were killed immediately after the fast, at every hour up to 8 hours, and at 12 and 24 hours after the provision of food. Access to food began either at 11 A.M. (day experiment) or 11 P.M. (night experiment), and were concluded at 11 A.M. or 11 P.M. the following day.

In the starvation experiments the animals (mice) fasted 24 hours, had free access to food for 12 hours, and then the food was removed and the animals killed every two hours up to 24 hours after its removal (see fig. 1). One group of experiments was begun at 11 A.M. and the other at 11 P.M.

One piece of each liver was used to determine the glycogen by the method of Nordenskiöld, Sjögren, Holmgren and Möllerström ('38). See also Swensson ('45).

Another piece was fixed in absolute alcohol for morphological studies of the distribution of the glycogen in the lobules. These pieces were fixed for 24 hours, imbedded in paraffin, cut into sections 5 μ thick and stained by Best's method. The best staining result was obtained after the sections remained in warm 70% alcohol at 45°–47°C. A number of sections from different parts of each liver were examined.

Evaluation of the sections

In evaluating the liver sections the following principles have been applied:

1. If the same quantity of glycogen appeared in all the cells in the lobule, it was called a diffuse type of deposit (fig. 7).

2. If glycogen appeared only in the area around the perilobular vessels and the bile ducts or if it appeared in clearly increased quantities in the peripheral half of the lobule, it was classified as a peripheral deposit (fig. 6). In cases where the accumulation of glycogen approached nearer the vena centralis, it was difficult to decide whether or not it was an augmented instance of what was really a peripheral glycogen deposit. In these doubtful cases livers were classified under the diffuse type.

3. In evaluating liver sections with accumulation of glycogen around the vena centralis, called the central type of deposit, the same principle was applied as in evaluating the peripheral (fig. 8).

The results of the experiments with mice prompted us to investigate glycogen metabolism of white rats with the aid of radioactive phosphorus. Twelve animals were allowed to fast for 24 hours, and then given food during a period of 4 hours before being at 2 P.M. or 2 A.M. On hour before being killed they had an interperitoneal injection of the same quantity of P^{32} in each experiment (see p. 196). As evidence that the animal had eaten, the stomach was weighed with and without its contents.

1. Experiments with free food after 24 hours fast

A. Day experiment. Table 1 shows that the average content of the stomach as percentage of body weight increases during the first hour from 0.38% to 4.21%. After that the increase is less pronounced except at 6 P.M. when the figure is figure 12.14%. The results give no indication of how much the animals have eaten, except that they have eaten food in considerable quantities.

The initial amount of glycogen is low, $0.29 \pm 0.05\%$ and corresponds with the long fast. As figure 1 shows, the glycogen rises almost in a straight line up to 7 P.M., that is, until 8 hours after access to food, at which time it is $9.44 \pm 0.65\%$. There is an additional insignificant increase until 12 hours after access to food ($9.74 \pm 0.67\%$). The full gly-

cogen capacity of the liver seems to have been reached at this time, because no important increase can be expected after 12 hours. On the following day at 11 A.M. when the experiment was concluded, the glycogen figure was 4.26 ± 0.35% in spite of free access to food. The table also shows that the liver weight increases in percentage of the body weight parallel with the increase of glycogen. This fact has been

TABLE 1a

Day animals

TIME	NUMBER OF HOURS OF FEEDING	NUMBER OF ANIMALS	WEIGHT OF LIVER AS % OF BODY WEIGHT	STOMACH CONTENT AS % OF BODY WEIGHT	GLYCOGEN AS % OF LIVER WEIGHT
11 A.M.	0	20	4.99	0.4	0.29 ± 0.05
12 P.M.	1	10	4.58	4.2	1.36 ± 0.27
1 P.M.	2	10	5.00	4.8	2.20 ± 0.27
2 P.M.	3	9	5.08	3.9	3.05 ± 0.31
3 P.M.	4	10	4.35	6.2	4.50 ± 0.33
4 P.M.	5	10	5.59	6.2	5.26 ± 0.29
5 P.M.	6	14	5.33	7.3	7.00 ± 0.40
6 P.M.	7	13	5.51	12.1	8.32 ± 0.48
7 P.M.	8	9	6.52	6.9	9.44 ± 0.65
11 P.M.	12	10	6.44	5.0	9.74 ± 0.67
11 A.M.	24	8	6.09	1.8	4.26 ± 0.35

TABLE 1b

Night animals

TIME	NUMBER OF HOURS OF FEEDING	NUMBER OF ANIMALS	WEIGHT OF LIVER AS % OF BODY WEIGHT	STOMACH CONTENT AS % OF BODY WEIGHT	GLYCOGEN AS % OF LIVER WEIGHT
11 P.M.	0	12	4.41	0.5	0.42 ± 0.08
12 P.M.	1	10	4.43	6.9	2.10 ± 0.22
1 A.M.	2	9	4.55	7.3	3.46 ± 0.22
2 A.M.	3	10	4.56	7.8	5.00 ± 0.43
3 A.M.	4	9	5.26	7.9	6.10 ± 0.25
4 A.M.	5	10	5.78	8.3	8.76 ± 0.92
5 A.M.	6	10	4.90	7.5	8.55 ± 0.51
6 A.M.	7	10	5.25	8.9	9.83 ± 0.73
7 A.M.	8	10	5.59	7.3	10.55 ± 0.50
11 A.M.	12	10	5.96	5.9	10.64 ± 0.76
11 P.M.	24	9	5.67	2.9	6.41 ± 0.35

noted earlier (Forsgren, '35; Holmgren, '36) but the figures of these day and night experiments are a clear confirmation of it.

Night experiment. This experiment was conducted in the same way as the day experiment, except for the time of killing the animals.

The content of the stomach increases during the first hour from 0.5% of the body weight to 7.8% and then remains fairly constant. In this experiment the animals seem to have eaten satisfactorily. It is not possible to determine whether they ate more in this experiment than in the previous one. but in any case the amount eaten should suffice for maximum storing of glycogen.

Figure 1 shows that in this experiment the liver glycogen increases, in general, in a straight line but after 5 hours an unexpectedly high value is seen. The reason for this is not clear. There is no reason for supposing that the value can be based on faulty analysis. The values are more widely spread than in the other groups as is shown by the average margin of variation. It is probable that, when the graph as a whole begins to reach its maximum, some of the animals reach a maximum storage of glycogen earlier than others, which results in less compact figures. In the group concerned there are three animals with a glycogen of 10%, a figure which usually does not appear until 7–12 hours after food. The high value 5 hours after the cessation of the fast in an average and probably expresses an accidental accumulation of high values.

The graph reaches its maximum ($10.64 \pm 0.76\%$) 12 hours after the insertion of the food. The 8 hour value ($10.55 \pm 0.50\%$) shows only an insignificant difference from this. A further point of interest is that the 24 hour value is $6.41 \pm 0.35\%$, which is consideraably higher than the corresponding value for the day experiment, $4.26 \pm 0.35\%$.

The graph of the night experiment follows the general lines of the graph for the day experiment, except that the night graph rises more steeply and the final 24 hour value is higher.

It is interesting to compare the two graphs in closer detail. A point by point analysis (table 2) shows that the variation between the values only 2 hours after inserting the food is statistically confirmed. This clear difference continues until 5 hours after the termination of the fast. After this, the difference for the times 7 and 8 hours after beginning the feeding is statistically probable. The values for 8 and 12 hours in the two graphs do not show a difference which is statistically certain. A point of particular interest is that the 24 hour values show a difference of 2.15%, $P<0.001$.

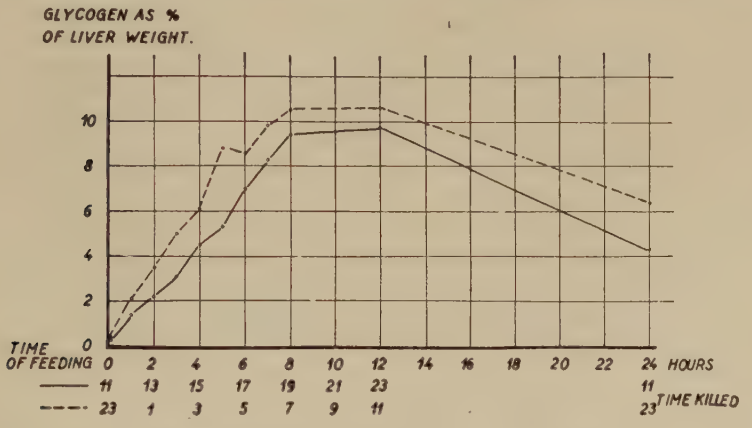


Fig. 1 Liver glycogen variations after 24 hours fast, followed by free access to food.

Figure 1 shows that the glycogen values in both experiments rise in an almost straight line until 8 hours after the termination of the fast. If the two graphs really show a slope difference in the rise in this area, it would mean that there is a difference in intensity in the storage of glycogen controlled by the time of day or night at which the experiments were conducted. To test this we have calculated the regression lines for the two parts of the graphs in question. Table 3 gives the results of this calculation.

The rectilinear regression line is a good approximation of the increase of the glycogen with values from zero to 8 hours,

both for day and night series, which is to be seen from the high correlation coefficients, 0.94 and 0.92 respectively.

The two lines differ significantly with regard to their means (< 0.001), and probably also with regard to their regression coefficients ($= 0.05 - 0.01$), calculated by means of analyses of covariance.

TABLE 2

NUMBER OF HOURS OF FEEDING	VARIATION	t	df	P
1	0.74	2.18	18	0.05 — 0.02
2	1.26	3.71	17	0.01 — 0.001
3	1.95	3.68	17	0.01 — 0.001
4	1.60	3.91	17	0.01 — 0.001
5	3.50	3.65	18	0.01 — 0.001
6	1.55	2.37	22	0.05 — 0.02
7	1.51	2.17	21	0.05 — 0.02
8	1.11	1.35	17	0.2 — 0.1
12	0.90	0.89	18	0.5 — 0.3
24	2.15	4.39	15	< 0.001

TABLE 3

	NUMBER OF ANIMALS	REGRESSION EQUATION	REGRESSION COEFFICIENT	CORRELATION COEFFICIENT	MEAN OF GLYCOGEN VALUES	
					Time	Per cent
Day animals 0–8 hours	105	$y = 1.14$	1.14 ± 0.04	0.94 ± 0.01	3.75 h.	4.36 ± 0.11
Night animals 0–8 hours	90	$y = 1.30$	1.30 ± 0.06	0.92 ± 0.01	3.93 h.	5.99 ± 0.16

Experiments with radioactive phosphorus

These experiments are to be regarded as a complement to the previous experiments with free access to food after 24 hours fasting. After 24 hours without food the rats had free access to food for 4 hours. The experiments were conducted both at 2 A.M. and 2 P.M. One hour before the animals were killed they were given an intraperitoneal injection of the same quantity of P^{32} in each experiment in the form of Na_2PO_4.

In pieces of liver the glycogen was determined as well as the total P and P^{32}, and the inorganic and organic phosphate (table 4). Phosphate determination was made by Brigg's ('22) method. A more detailed description of the analysis is found in an earlier work (Ekman and Holmgren, '47). The radioactivity was determined by Geiger-Müller counters (Levi, '41) up to 1000 impulses. The number of impulses per minute were counted with correction made for the zero number and the inertia of the counter.

Before going into details of our results, it is of interest to consider what intermediary carbohydrate products in the view of various writers ought to be present in the 10 and 180 minute hydrolyses. In an investigation of the ATP (adenosine triphosphate) of the pyrophosphate group and its metabolism in the cell, it was found (Lohmann, '28) that from ATP during hydrolysis in N HCl during 0–180 minutes, the greatest quantity of phosphate was detached after 10 to 15 minutes. If the hydrolysis is continued up to 180 minutes, the figure rises insignificantly. Cori and Cori ('36) maintain that in trichloracetic acid extract of muscle there is 34% of ATP and that if the extract is hydrolysed with N HCl for 7 minutes at 100°C., 100 gm of muscle gives 69 mg of ATP. Investigations (Meyerhof et al., '38) of the glycolysis with the help of radioactive phosphorus has also shown that in a 7 minute hydrolysis the pyrogroups are obtained in ATP. Kaplan and Greenberg, '43, '44, have studied the carbohydrate replacement in the liver and muscles with the help of P^{32}. They precipitated the trichloracetic acid extract with $Ba(OH)_2$ at pH 8.2, and in the course of the precipitation determined various phosphate fractions. These writers maintain that with 7 minutes hydrolysis in N HCl they obtain the two unstable phosphate groups in ATP. After further precipitation of the hydrolysis they determined ribose phosphate and the stable phosphate group in ATP. It is of interest that these writers maintain that the amount of light hydrolysable phosphate in the liver extract which does not come from ATP is insignificant.

TABLE 4

TIME	WEIGHT OF ANIMAL IN GM	WEIGHT OF LIVER IN GM	GLYCOGEN AS % OF LIVER WEIGHT	LIVER FOR ASHING		SPECIFIC ACTIVITY						
						Piece of liver		Trichloracetic acid extract				
				^{32}P/100 mg	P/100 mg	Total phosphate	Non-acid-soluble phosphate	Inorganic phosphate	10 'H	180 'H	Residue fraction	Total phosphate in the extract
2 P.M.	71	3.09	2.43	435	354	12.3	2.0	61.5	20.5	10.1	23.6	41.9
2 P.M.	71	2.70	1.55	576	354	16.3	6.1	64.0	28.0	16.7	21.9	40.5
2 P.M.	93	3.73	1.66	340	264	12.9	2.7	53.4	20.0	20.0	13.0	29.0
2 A.M.	86	3.26	3.57	389	264	14.7	3.6	56.8	47.2	17.9	20.0	37.0
2 A.M.	87	3.15		462	264	17.5	8.8	70.7	32.8	10.3	8.9	37.6
2 A.M.	84	3.76	3.89	425	252	16.9	6.7	65.4	33.3	25.6	34.1	38.5
2 P.M.	104	4.1	2.02	358	336	10.6	3.6	32.9	20.8	10.3	24.0	25.2
2 P.M.	127	5.2	2.95	333	342	9.8	2.8	32.0	12.0	7.0	16.4	23.6
2 P.M.	104	4.0	2.12	350	362	9.7	2.2	33.6	11.8	13.5	16.9	23.6
2 A.M.	80	3.1	4.01	430	329	13.0	2.5	41.0	33.8	14.0	21.6	31.4
2 A.M.	110	5.1	3.88	409	387	10.5	3.1	34.5	25.0	12.4	21.0	27.0
2 A.M.	102	4.8	3.91	400	452	8.8	2.6	42.7	24.6	12.4	17.4	30.0
2 P.M.	Average		2.12	397	335	11.9	3.2	51.9	18.8	12.9	19.3	30.6
2 A.M.	Average		3.87	419	325	13.6	4.5	46.2	32.8	15.4	20.5	33.6

In the 180 minute hydrolysis a series of different carbohydrate products are obtained, such as hexosediphosphate, Robinson-ester, glucose-6-phosphate, fructose-6-phosphate, phosphor-glycerine acid, etc. (see Bamann and Myrbäck, '41).

It appears from the literature that hydrolysis of trichloracetic acid extract gives off phosphates from various intermediary carbohydrate products.

The results of our investigations are shown in tables 4 and 5 where table 5 shows the average figures for γ P/100 mg of liver in animals killed at 2 P.M. and 2 A.M. It will be seen that these figures do not give any basis for a difference in the phosphate content between the two groups of animals, even in spite of the fact that the difference (1.75%) in liver

TABLE 5

P/100 mg liver

TIME	NUMBER OF ANIMALS	INORGANIC PHOSPHATE	10 'H	180 'H	ACID-SOLUBLE PHOSPHATE	TOTAL PHOSPHATE	NON-ACID-SOLUBLE PHOSPHATE
2 P.M.	6	52.9	17.4	19.4	107.9	397	227.5
2 A.M.	6	44.6	15.0	19.1	95.8	325	228.9

glycogen content in animals killed at 2 P.M. and 2 A.M. is statistically significant ($P < 0.001$).

A question of greater interest is the behaviour of the specific activity ($\frac{P^{32}}{P} \cdot 100$). Calculated in this way P^{32} gives for the 10 minute hydrolysis a certain difference between the two groups of animals. The figure for the 2 P.M. group is 18.8 and for the 2 A.M. group 32.8. The difference of 14.0 is definite ($P < 0.001$). Both with the 180 minute hydrolysis and the other fractions, the differences are insignificant and cannot be defined.

The experiments show that with very heavy feeding at 2 P.M. and 2 A.M. after fasting previously, the glycogen reaches a higher figure in the latter case. This fact is also reflected in the specific activity in the 10 minute hydrolysis. Since

the 10 minute hydrolysis consists largely of ATP and since this is part of the reversible system which seems to be the central mechanism for transference of energy from exergonic to endogonic reactions in carbohydrate metabolism, the higher specific activity for the night animals presumably means an increased carbohydrate metabolism.

The results apparently contradict earlier observations on normal animals (Ekman and Holmgren, '47), which show that the metabolism of the marked phosphorus in the carbohydrate fractions is more active during the day than during the night. The explanation must lie in the fact that the latter experiments are conducted with animals which have free access to food where both glycogenesis and glycogenolysis occur. In the present work, on the other hand, it is principally a question of accumulating glycogen. The glycogen values show that this is most pronounced in the night animals, which result agrees well with the greater yield of marked phosphorus in the 10 minute hydrolysis of liver from these animals.

Starvation experiment

The two starvation experiments (table 6 a and b) were conducted in a similar manner except that one took place during the day and the other during the night. In addition, we discontinued the night experiment after 12 hours starvation, the day experiment after 24 hours.

Figure 2 shows that during the day (table 6 a) the glycogen value sinks slowly until 5 P.M., that is, 6 hours after the commencement of the starvation. The variation, 2.17%, between the initial 11 A.M. value and the 5 P.M. value is the first to be statistically probable, $P = 0.01 - 0.02$, so that no result of the starvation can be read off from the earlier glycogen values. It seems, therefore, that the liver glycogen begins to sink noticeably only after 6 hours without food. Whether this decrease is due only to the starvation or is also influenced by the decrease in liver glycogen which normally takes place at this time of day (Ågren, Wilander and Jorpes, '31), cannot be determined for certain.

The difference (3.33%) between the initial value and the 8 hour value is still more pronounced, $P = 0.01 - 0.001$. The glycogen falls almost in a straight line to a minimum ($0.20 \pm 0.02\%$) 18 hours after the beginning of the starvation and rises again slightly until 9 A.M. (22 hours starvation). The variation (0.42%) between these two values can be statis-

TABLE 6a

Starvation animals

Day experiment

TIME	NUMBER OF HOURS STARVATION	NUMBER OF ANIMALS	WEIGHT OF LIVER AS % OF BODY WEIGHT	STOMACH CONTENT AS % OF BODY WEIGHT	GLYCOGEN AS % OF LIVER WEIGHT
11 A.M.	0	14	5.91	5.1	7.23 ± 0.52
1 P.M.	2	10	6.34	0.8	6.03 ± 0.32
3 P.M.	4	10	5.10	0.6	5.17 ± 0.83
5 P.M.	6	10	5.26	1.0	5.06 ± 0.89
7 P.M.	8	10	4.88	0.6	3.90 ± 0.74
9 P.M.	10	10	4.81	0.3	2.60 ± 0.46
11 P.M.	12	10	4.44	0.3	1.89 ± 0.40
1 A.M.	14	10	4.05	0.5	0.79 ± 0.22
3 A.M.	16	8	3.75	0.4	0.24 ± 0.06
5 A.M.	18	7	3.81	0.3	0.20 ± 0.02
7 A.M.	20	10	4.53	0.4	0.48 ± 0.07
9 A.M.	22	10	4.38	0.3	0.62 ± 0.05
11 A.M.	24	10	4.54	0.3	0.49 ± 0.11

TABLE 6b

Starvation animals

Night experiment

TIME	NUMBER OF HOURS STARVATION	NUMBER OF ANIMALS	WEIGHT OF LIVER AS % OF BODY WEIGHT	GLYCOGEN AS % OF LIVER WEIGHT
11 P.M.	0	5	6.43	7.50 ± 0.14
1 A.M.	2	5	6.91	8.13 ± 1.55
3 A.M.	4	5	6.01	6.40 ± 0.22
5 A.M.	6	5	6.10	5.69 ± 0.38
7 A.M.	8	5	5.71	5.63 ± 0.22
9 A.M.	10	5	5.81	5.23 ± 0.20
11 A.M.	12	5	5.63	3.01 ± 0.83

tically ascertained with a P-value of < 0.001. This small increase of liver glycogen has also been observed by others (see Swensson, '45). How far it is connected with the cyclic alterations of liver glycogen is difficult to determine.

In any case, the experiment shows that the effect of starvation on the glycogenolysis which normally occurs during the day is not noticeable until 6 hours after the removal of the food, a fact which agrees well with work of Neufeld and Collin and others ('41) and Swensson ('45).

Table 6 b gives the values for the starvation experiments during the night. The initial value of $7.50 \pm 0.14\%$ is about

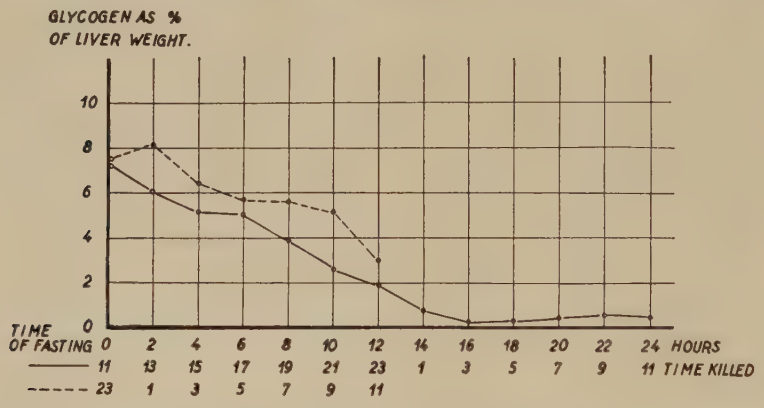


Fig. 2 Liver glycogen variations in rats during prolonged starvation. (For nature of the experiments, see p. 191.)

the same as the one for the day experiment. After one hour without food the graph (table 6 b) shows a rise which, with the wide margin, does not indicate any real increase of liver glycogen. As starvation continues the graph falls parallel with that of the day experiment. It is remarkable, however, that all the values for the night experiment are higher that those of the day experiment. As the former are based on only 30 animals it is hardly possible to show any difference between the rectilinear regression line for the two experiments. In spite of this it is of great interest to know whether the variations between the points on which

the two graphs are based show a difference which is statistically certain. The variations between corresponding values from 1 hour to 12 hours starvation in the two graphs are all positive, that is to say, the values of the night experiment are higher. If a t-analysis of these variations is made, it gives a t-value of 6.54 ($P = 0.01 - 0.001$).

The statistical calculation thus shows that the variation certainly differs from 0. This fact points strongly to a systematic variation between the two graphs, consisting of a higher glycogen value in livers from animals killed during the night.

DISCUSSION

The question of the importance of alimentary factors must obviously be a central problem in the evaluation of investigations into the cyclic variations of liver glycogen. In our experiments we have tried to create starting points as similar as possible for the various experiments. Only the time for carrying them out has been varied. By this arrangement we have tried to show that there is a factor of importance for the storage of glycogen outside the purely alimentary conditions.

It is of importance for the experiments involving feeding that the mice really have eaten. When the animals have been without food for 24 hours before the experiment, they have naturally begun to eat as soon as they have been given food, as is shown by the weight of the content of the stomach. It is obvious that the values differ individually and for the different groups of animals, depending on whether or not the animal has eaten as much just before it was killed. Besides it is easy to lose varying amounts of the contents of the stomach when removing the organ. In any case there seems no reason to suppose that the animals have not eaten enough food to make possible a maximum deposit of liver glycogen. This means that in both experiments there must have been the same alimentary preconditions. From that point of view the different course of the graphs from zero to 8 hours after the end of the fast is of interest (fig. 2). The statistical analy-

sis shows that the difference between the graphs is not accidental. As the experiments have been carried out under uniform conditions: the stock, sex, temperature, the time of year and alimentary factors, it seems probable that the difference between the experiments is connected with their being conducted at different times of day. The experiments show that there is a greater intensity in the storage of glycogen in the case of the night animals than in that of the day animals. If we consider the glycogen values 24 hours after the end of the fast further we see a difference, statistically significant, with a higher value for the night group (11 P.M.) than for the day group (11 A.M.).

This difference in principle between the day and night animals is demonstrated in another way by our experiments with P^{32}. The glycogen value of the night animals is higher than the corresponding value for the day animals. In addition, the yield of marked phosphorus in the 10 minute hydrolysis is larger than in the former group. The experiments with P^{32}, carried out with the same conditions as the experiments with mice except for the alteration of the time for the experiments, verify the results of the latter experiments.

Our experiments indicate, therefore, that in normal animals there is a difference in the intensity of glycogen storage, associated with the time of day. This difference is not dependent on the amount of food in the alimentary canal but the time of day the food is given. This confirms the principle of a normal cyclic variation in the quantity of liver glycogen. Whether the cause of the rhythmic variations is to be found in the liver itself or whether it is dependent on a cyclic absorption from the intestine or whether it is controlled by nervous or endocrine activity, we prefer to leave unsaid.

Histophysiological section

Since Bock and Hoffman in 1872 pointed out that liver glycogen in rabbits is first deposited around the vena centralis, a number of research workers (see Holmgren, '36) have been interested in the localization of glycogen in the

liver lobule. The distribution of glycogen has been investigated in the livers of human beings and a number of animals. Different types of deposit have been observed, some diffuse over the whole lobule, some in the peripheral and some in the central zones. Some authors consider that the differences are connected with the cause of death (Klestadt, '11; Meixner, '11). It has also been suggested that differences in the circulation in the lobule is the ultimate cause of the varying pattern (Klestadt, '11). Finally there are those who hold that the different types of deposit represent various functional stages. In accordance with this functional theory it has been held (Laquer, '22) that glycogenolysis begins at the periphery, producing a pattern of central deposit. With rats (Smith, '31) the deposition starts with the periphery and moves towards the center, but with mice the reverse is the case. It is of interest that, after intravenous injection, glycogen was seen in the periphery of the lobule (Horsters, '32). In rabbits the cells of the peripheral zone are mainly concerned with the formation of the early stages of the bile while the cells of the central zone are more engaged with glycogen deposit (Forsgren, '35, '27). With rats under the influence of adrenaline and insulin, glycogen deposit begins in the periphery of the lobule and moves toward the center. A purely central deposit pattern points to glycogenolysis (Edlund and Holmgren, '40).

Deane ('44) studied glycogen deposit in the lobules of white mice. Her material consisted of 8 animals on free food and also of a group of 16 males and 16 females which were fed between 7 A.M. and 10 A.M. for 4 days before they were killed. In the former series one animal was killed every three hours during the day and night. In the second series every three hours up to 24 hours after the last feeding. In the first series — uncontrolled diurnal cycle — animals killed between 12 noon and 3 P.M. had a peripheral deposit of glycogen. In animals killed between 6 and 9 P.M., on the other hand, "the few granules of glycogen are most concentrated in the central zone." In the other series — controlled diurnal cycle —

Deane found a diffuse distribution in the lobules of most of the animals. Only in the liver of animals killed 18–21 hours after feeding "it may be more concentrated in the peripheral part of the lobule" and in only one mouse 21 hours and one other 24 hours after feeding was the glycogen concentrated in the central zone.

On the basis of her very limited material Deane considers that glycogen deposit begins in the peripheral cells and later central cells begin gradually to fill. Glycogenolysis, on the other hand, begins in the central zone, according to Deane, because in this zone there is a relative anoxia and according to Mathews ('39) "glycogen is immediately broken down to sugar under anaerobic conditions." The glycogenolysis in the center, however, is not complete. It is followed by a total peripheral, glycogenolysis with the result that there remains a central deposit of glycogen. Why the glycogen does not completely disappear in the cells in the central zone despite the supposed anoxia of this region, she is unable to say.

Results of experiments

Our study includes the distrbiution of glycogen in the liver lobule in day and night feeding experiments and in starvation experiments. The former deal principally with glycogen deposit, the latter mainly with a glycogenolysis, representing two different phases in the work of the liver.

Experiments with food after 24 hours fast

The results are shown graphically in figures 3 and 4. One hour after the insertion of food the glycogen in almost all the livers investigated is stored peripherally in the lobule (figs. 3 and 4), although the size of the zone differs from one liver to another. The cells in the central part of the lobule are as a rule free of glycogen observable with a microscope. In only two animals in the day experiment the rows of cells around the vena centralis contained fine grains of glycogen while farther out towards the periphery the cells had more

glycogen in the form of large irregular lumps. These animals have been included among those having the peripheral type of deposit.

Two hours after the animals have eaten, diffuse patterns of glycogen first appear. No difference can be determined with the microscope between the amount of glycogen in the peripheral and central areas. The number of livers with diffuse glycogen increases gradually, while the number with peripheral deposit in general decreases. In the night experi-

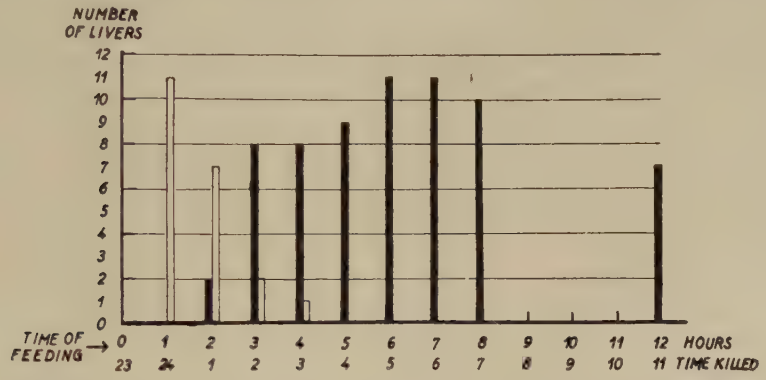


Fig. 3 Number of livers with peripheral and total deposit in the lobuli in animals killed at different times after the insertion of food (night experiment).
White columns = peripheral deposit.
Black columns = total deposit.

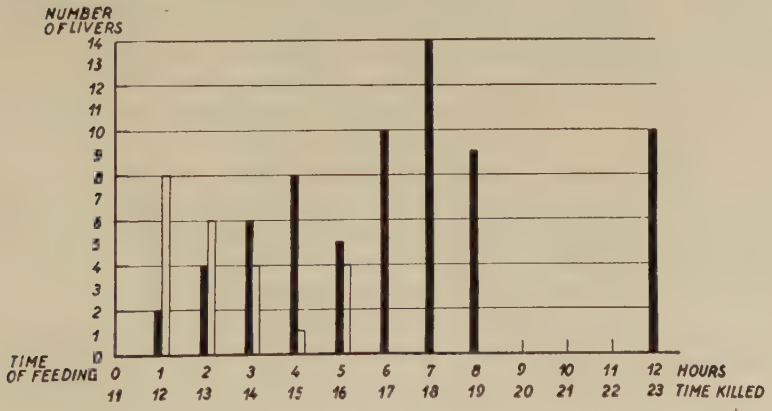


Fig. 4 Day experiment. For explanation, see figure 3.

ment, 5 hours after the insertion of food all livers had a diffuse distribution while in the day experiment this distribution was reached only after 6 hours. Twelve hours after breaking the fast only livers with a diffuse distribution of glycogen were found.

In animals killed 24 hours after the insertion of food the livers of those killed at 11 P.M. all have diffuse distribution.

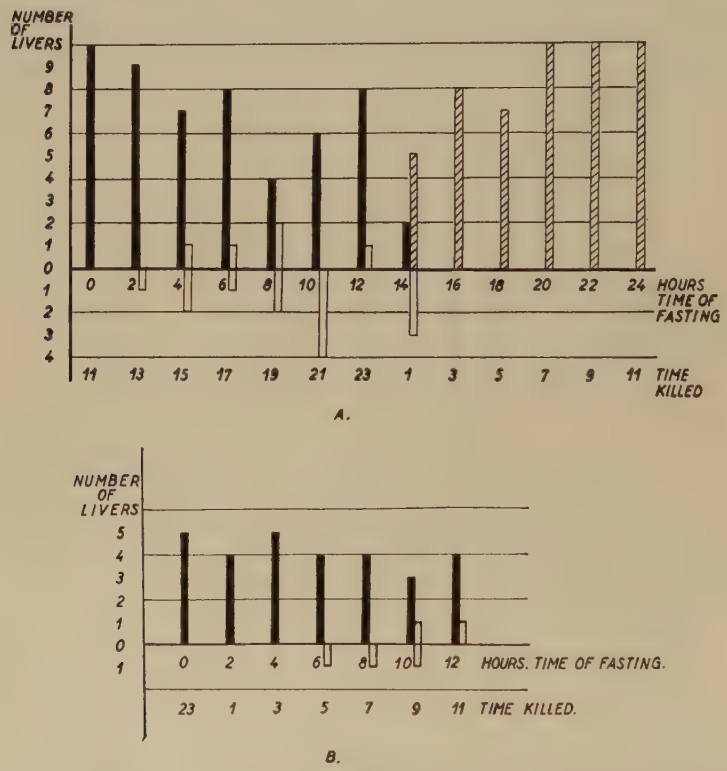


Fig. 5 Number of livers with central and total deposit in the lobuli, and livers devoid of colorable substance in animals killed after starvation of varying length.

White columns downwards = central deposit.
White columns = peripheral deposit.
Black columns = total deposit.
Shadowed columns = no colorable substance.

A. Day experiment.
B. Night experiment.

Livers of mice killed at 11 A.M. on the other hand give 4 with diffuse distribution and three with a central deposit, out of 7 investigated.

To investigate further where the accumulation begins, 7 mice were given an intraperitoneal injection of 0.5 ml 10% glucose after 24 hours fasting. After a further hour and a

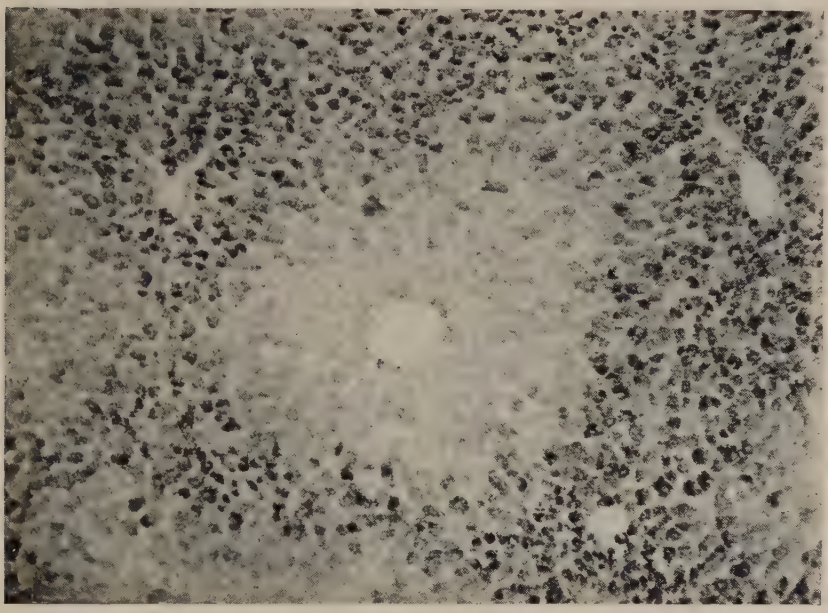


Fig. 6 Peripheral glycogen deposit in the lobuli of the liver in animals that had fasted for 24 hours and then received an intraperitoneal injection of 10% glucose. Killed 1½ hours after injection.

Thickness of section = 5
Coloring = Best's carmine staining
Enlargement = 125

half the animals were killed (three animals that had fasted but not been injected were used as controls). In every animal the glycogen was found deposited in the periphery of the lobule. In some animals the distribution was not pronounced, but in other cases it was clearly marked (fig. 6). The livers of the three controls were devoid of glycogen which

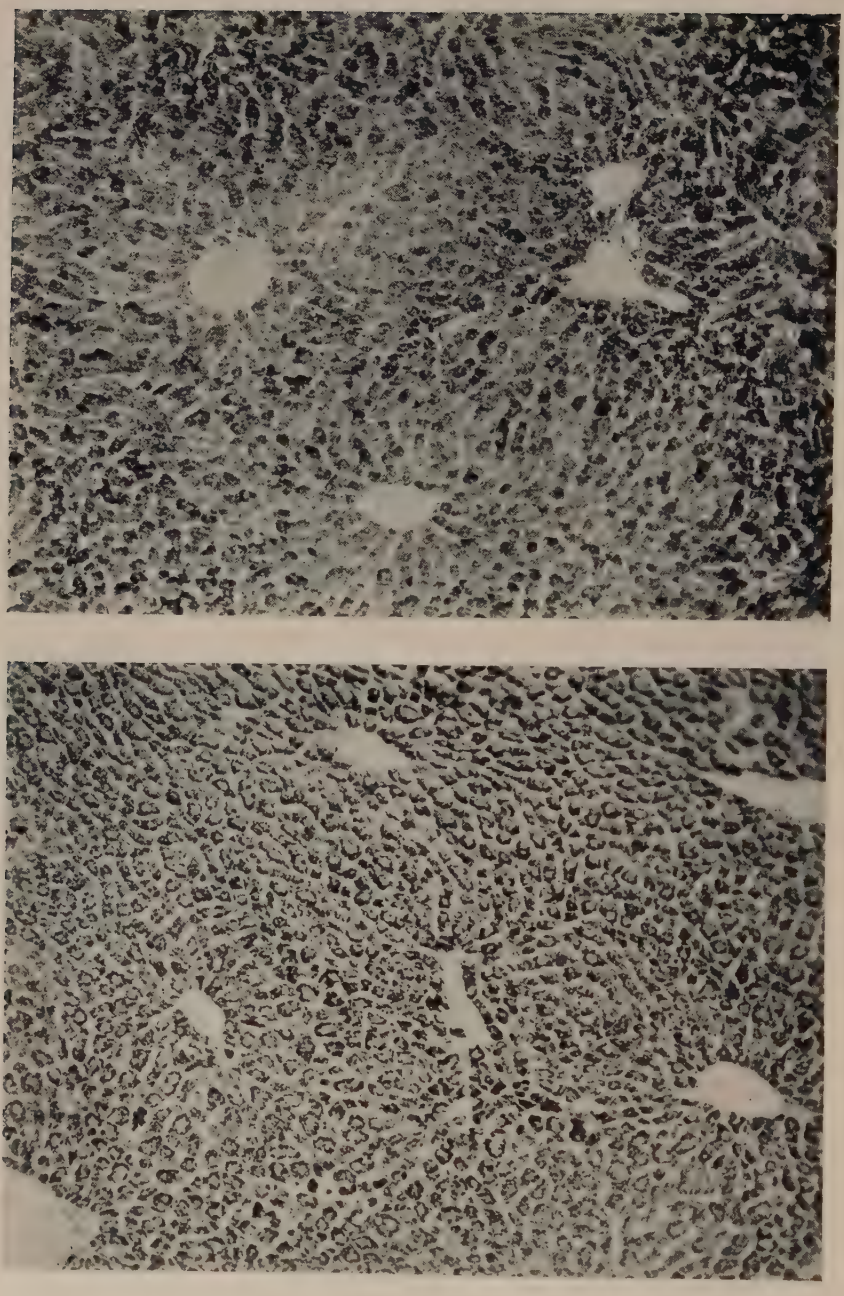


Figure 7

A. Peripheral deposit of glycogen. Animal killed at 12 midnight, one hour after insertion of food. Glycogen content, 1.59%.

B. Total glycogen deposit. Animal killed at 11 P.M., 12 hours after insertion of food. Glycogen content 5.94%.

could be observed microscopically. The experiment indicates that accumulation of glycogen begins in the peripheral zone of the lobule.

The starvation experiments

The day experiment (11 A.M. to 11 P.M.). The distribution of the glycogen in the lobule is shown in figure 5 a and b.

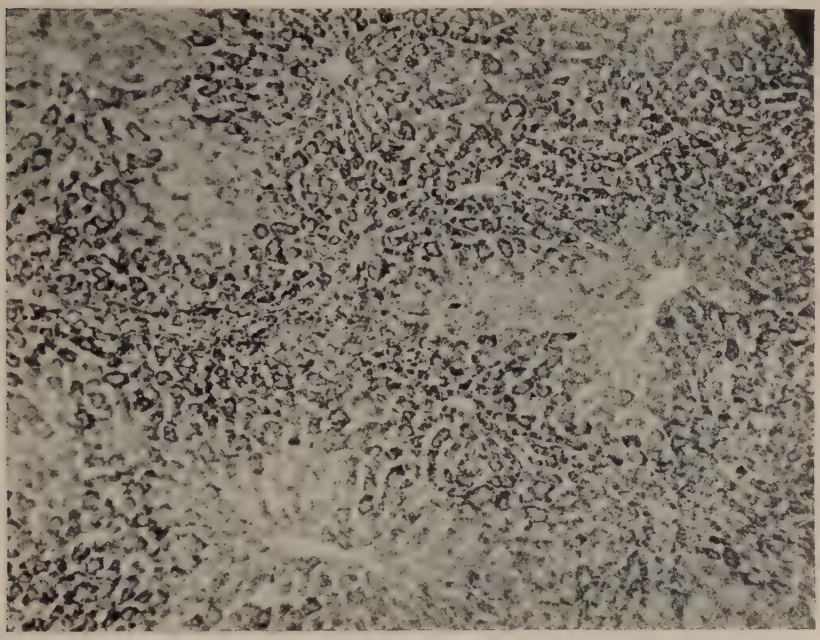


Fig. 8 Central glycogen deposit in the lobuli. Animal killed at 3 P.M. after 4 hours fasting. Glycogen content 4.93%.

For treatment, see figure 7.

At the time of removal of food at 11 A.M. all livers have the same diffuse pattern of deposit (fig. 7 b). After 2 hours one liver has a clear accumulation of glycogen around the vena centralis (fig. 8). As the fast continues livers with central type of deposit occur more and more often, but at the same time an occasional liver appears with apparently peripheral deposit. Only after 14 hours are all livers practically devoid

of stainable glycogen. In occasional cells, however, it is still possible to find fine crimson lumps, similar in appearance to lumps of glycogen in livers with a rich glycogen content. In livers with an analysis figure under 0.3 to 0.5% glycogen, we have never found any certain glycogen in the sections.

The night experiment (11 P.M. to 11 A.M.): In this experiment the glycogen patterns differ a little from the day experiment. The majority of livers have a diffuse pattern. In three animals we observed a central glycogen deposit and in two we found a suggestion of peripheral accumulation 10 and 12 hours after the beginning of the fast.

DISCUSSION

Our experiments show that on the insertion of food after 24 hours fast, glycogen begins to appear in the periphery of the lobule (fig. 7). This must be due to the fact that the cells in this part of the lobule are the first to come into contact with the nutritive portal blood. This type of deposit occurs also in the great majority of livers one hour after the insertion of food. It seems clear to us, therefore, that a peripheral pattern of deposit is a sign of glycogen synthesis, a view which agrees well with the results of other research workers. It is of interest that in the day experiment there are still livers with peripheral deposit 5 hours after the ingestion of food, while in the night experiment this type is not represented at all at that time. Whether this is accidental or whether it is due to faster filling of the lobule at night, is difficult to determine. Since the glycogen graph for the day experiment rises more slowly than the corresponding graph or the night experiment, it seems to support the contention that the distribution of livers with a peripheral type of deposit is dependent on the speed with which glycogen synthesis occurs.

The patterns of deposit which appear 24 hours after the ingestion of food are of interest. At 11 P.M. the livers contain on an average 6% and at 11 A.M. 4% glycogen. Since this variation must depend on the normal cyclic glycogen varia-

tions, chiefly glycogen storage in the former case and chiefly glycogenolysis in the latter, it is of interest to see whether this is reflected in the distribution of glycogen in the lobule. This seems to be the case. All the livers of animals killed at 11 P.M. have diffuse storage, whereas, on the other hand, three of those killed at 11 A.M. have a central storage pattern. This is probably due to glycogenolysis being so advanced in the latter case that it is observable in the slide. That a central deposit pattern can express glycogenolysis is proved also by the starvation experiments. With regard to these, in the day experiment we have found livers with both central and peripheral glycogen distribution. In the night phase, with its higher glycogen figures, most of the livers have a diffuse deposit and only two of them a peripheral. This would seem to point to a central pattern being a sign of glycogenolysis since in the other experiment where there is mainly glycogen synthesis we have found only livers with peripheral or diffuse deposit. In spite of this, it is possible for a liver from an animal that has been starving to have a peripheral pattern. Our pictures then must mean that the position of glycogen within the lobule during glycogenolysis in mice does not follow the fixed scheme that glycogen deposit follows. In any case an anoxia in the central parts of the lobule can hardly be decisive, as Deane holds, for the position of glycogen during glycogenolysis.

Since the lobules differ greatly in form and size, it seems possible that the direction of the slicing may cause an apparent peripheral distribution. If this were so, we should find both central and peripheral distribution in the lobules on the same slide, just as both types should be represented in our experiments with free access to food. The patterns of peripheral deposit which we have found in the starvation experiments are not as unequivocal as in the experiments with food. But the second fact casts doubt on this explanation. In spite of this we cannot wholly disregard the possibility that the form and size of the lobules and their position with regard to one another can give wrong pictures of the distribution

of glycogen. It may be concluded from our observations that glycogen deposit begins in the periphery of the lobule and continues towards the center. Only after all cells in the lobule as a whole contain the same amount of glycogen is glycogenolysis made visible by the loss of glycogen beginning in the peripheral cells. In this way the central pattern of deposit may be supposed to occur. That the peripheral type can be due to central glycogenolysis, caused in its turn by a relative anoxia in this region, as Deane propses, seems to us unlikely. Her pictures are not convincing, and her limited material allows us to form no certain conclusion as to the course of glycogen deposit in the lobule.

SUMMARY

To study the importance of alimentary factors for cyclic glycogen variations in the liver and to get a picture of glycogen deposit in the lobule, feeding and starvation experiments were carried out with white mice and rats.

1. Storage of glycogen after 24 hours starvation takes place faster during the night than during the day. The difference can be verified by statistics. Maxium deposit is reached 8 hours after the insertion of food. Even 24 hours after the end of fasting the glycogen values for the day animals are lower than those for night animals. The difference is 2.15% ($P < 0.001$).

2. In the experiments to follow the course of carbohydrate metabolism with the help of P^{32} during full feeding after 24 hours previous fast, it was possible to demonstrate a richer yield of marked phosphorus in the night animals than in the day animals.

3. If mice are put on starvation after 12 hours previous feeding, lowering of the glycogen content of the liver cannot be ascertained until after 6 hours.

4. A histological analysis of glycogen distribution in the lobule shows that glycogen deposit begins in the peripheral areas. After 4 hours all the liver cells contain glycogen which can be demonstrated histologically. Glycogenelysis usually

shows first as a decrease of glycogen in the periphery of the lobule, leaving a central type of deposit, but in certain livers the picture is questionable.

LITERATURE CITED

ÅGREN, G., O. WILANDER AND E. JORPFS 1931 Cyclic changes in glycogen content of liver and muscles of rats and mice; their bearing upon sensitivity of animals to insulin, and their influence on urinary output of nitrogen. Biochem. J., *25:* 777.

BAMANN UND MYRBÄCK 1941 Die Merhoden de Fermentforschung. Leipzig.

BOCK UND HOFFMAN 1872 Über das mikrochemische Verhalten der Leberzellen. Virchows Arch., *56:* 201.

BRIGG, A.P. 1922 A modification of the Bell Doisy phosphate method. J. Biol. Chem., *53:* 13–16.

CORI, G. T., AND C. F. CORI 1936 Formation of hexosephosphate esters in frog muscle. J. Biol. Chem., *116:* 119.

DEANE, H. W. 1944 Cytological study of diurnal cycle of liver of mouse in relation to storage and secretion. Anat. Rec., *88:* 39.

EDLUND, Y., UND HJ. HOLMGREN 1940 Zur Kenntnis der Lokalisation des Leberglykogens unter Adrenalin-beziehugsweise Insulinwirkung. Ztschr. f. mikr. anat. Forsch., *47:* 467.

EKMAN, C.-A., AND HJ. HOLMGREN 1947 An investigation of the rhythmic metabolism of the liver with the help of radio-active phosphorus. Acta med. Scand., Suppl. 196.

EULER, v. U. S., AND A. G. HOLMQUIST 1934 Tagesrhythmik der Adrenalinsekretion und des Kohlehydratstoffwechsels beim Kaninchen und Igel. Arch. f. d. ges. Physiol., *234:* 210.

FORSGREN, E. 1927 Mikroskopiska och experimentella leverundersökningar. Diss. Stockholm.

———— 1935 Über die Rhythmik der Leberfunktion, des Stoffswechsels und des Schlafes. Acta Soc. med. Suecanal., *62:* 1.

GRAFFLIN, A. L., MARBLE, A. AND R. M. SMITH 1940 Glycogen, fat and water content of guinea pig liver. J. Biol. Chem., *134:* 253.

———— 1941 Note on histological estimates versus chemical analysis of liver glycogen. Anat. Rec., *81:* 45.

HAMAR, N. 1941 Über Tagesschwankungen des Glucoseresorptionsvermögens des Dünndarms. Pflügers Arch., *244:* 164.

HIGGINS. G. M., J. BERKSON AND E. FLOCK 1932 Diurnal cycle in liver; periodicity of cycle, with analysis of chemical constituents involved. Am. J. Physiol., *102:* 673.

———— 1933 Diurnal cycle in liver of white rat; food, factor in its determination. Am. J. Physiol., *105:* 177.

HOLMGREN, HJ. 1936 Studien über 24-stundenrhythmische Variationen des Darm,-Lungen und Leberfetts. Acta med. Scand., Suppl. 74.

HORSTERS, H. 1932 Physiologie und Pathologie der Galle. Erg. Physiol., *34:* 494.

JORES, A. 1935 Physiologie und Pathologie der 24-Stunden-Rhythmik des Menschen. Erg. d. inn. Med., *48:* 574.

KAPLAN, N. O., AND D. M. GREENBERG 1943 Observations with P^{32} of changes in acidsoluble phosphates in liver coincident to alterations in carbohydrate metabolism. J. Biol. Chem., *150:* 479.

———— 1944a Effect of starvation and agening on acid-soluble phosphate components of liver. Proc. Soc. Exp. Biol. and Med., *57:* 130.

———— 1944b Studies with radioactive phosphorus of the changes in the acid-soluble phosphates in the liver coincident to alterations in carbohydrate metabolism. J. Biol. Chem., *156:* 511.

KLESTADT, W. 1911 Über Glykogenablagerung. Erg. allg. Path. *15*: 2, 349.

KATER, J. M. 1933 Comparative and experimental studies on cytology of liver. Ztschr. f. Zellforsch. u. mikr. Anat., *17:* 217.

LAQUER, F. 1922 Die Stellung der Leber im intermediären Stoffwechsel. Klin. Wochenschr., *1:* 822.

LEVI, H. 1941 Geiger counter arrangement applied to measurements of radioactive indicators in biological research. Acta physiol. Scand., *2:* 311.

LOHMANN, K. 1928 Über das Vorkommen und den Umsatz von Pyrophosphat in Zellen. Biochem. Zeitschr., *202:* 466.

MATHEWS, A. P. 1939 Physiological chemistry.

MEIXNER, K. 1911 Das Glycogen der Leber bei verschiedenen Todesarten. Beitr. zu gerichtl. Med., *1:* 222.

MEYERHOF, O., P. OHLMEYER, W. GENTER UND H. MAIER-LEIBNITZ 1938 Studien der Zwischenreaktionen der Glykolyse mit Hilfe von radioaktiven Phosphor. Biochem. Zeitschr. *298:* 396.

NORDENSKIÖLD, T., B. SJÖGREN, HJ. HOLMGREN UND J. MÖLLERSTROM 1938 Beitrag zur Kenntnis der Leberrhythmik. Pflügers Arch., *240:* 427.

PETRÉN, T. 1939 Die 24-Stunden-Rhythmik des Leberglykogens bei Cavia cobaya nebst Studien über die Einwirkung der ''chronischen'' Muskelarbeit auf diese Rhythmik. Morph. Jahrb., *83:* 256.

SJÖVALL, E. 1912 Leberglycogen und gerichtliche Medizin. Vierteljahrschr. gerichtl. Med., *43.*

SWENSSON, A. 1945 Contributions to the knowledge of the effect of exogenous insulin on the glycogen storage of normal animals. Acta medica, Suppl. 33.

SMITH, D. 1931 The deposition and withdrawal of the glycogen of the liver in the rat. Anat. Rec., 51, Suppl. 74.

LYMPHOCYTES IN THE NORMAL EPIDERMIS OF THE RAT AND OF MAN

WARREN ANDREW AND NANCY V. ANDREW

Departments of Anatomy, Washington University School of Medicine, St. Louis, Missouri and The George Washington University School of Medicine, Washington, D. C.

ELEVEN FIGURES

The lymphocytes are notable for their great numbers and wide distribution in the body. Not only do they compose the bulk of entire organs, but as solitary or aggregated nodules, and in the form of diffuse lymphoid tissue, they underlie much of the mucosa of the alimentary and respiratory systems. As isolated cells, they may occur almost anywhere in the connective tissue.

If the hemocytoblasts of the bone marrow are identical with the large lymphocytes of the lymphoid tissue, this means the presence of great numbers of lymphocytes throughout the hematopoietic organs. Maximow and Bloom ('48, p. 107), supporting the unitarian theory of origin of all blood cells of the adult from one common type, take this view.

Of particular interest is the manner in which lymphocytes can be seen to migrate into epithelial layers in various locations. In some places, as in the tonsils and on the villi, and in the crypts of Lieberkuhn in the intestine, this migration is a conspicuous phenomenon. It has been shown, however, to occur in very many localities where more careful observation is required for the detection of the wandering cells. Schaffer ('27) lists the places where lymphocyte migration into epithelium has been reported. They include: oesophagus, intestine, trachea, various glands and their ducts,

uterus, oviduct, vagina, ureter and bladder, tongue, upper lip, epididymis, cloacal epithelium (in rays) and epidermis (in a fish, *Cobitis*).

In previous investigations we have noted different types of behavior of the lymphocytes in the epithelial layers of different organs. In the intestine, for example (Andrew and Andrew, '45; Andrew and Collings, '46; Andrew and Sosa, '47), they frequently enter into the epithelial cells and there undergo changes either degenerative or proliferative in character, the degenerating ones being cast into the lumen. In the trachea, on the other hand (Andrew and Burns, '47), they almost always remain intercellular, seldom show marked changes in the nuclei, and seem to remain in the epithelial layer, rarely reaching the lumen.

The epidermis, a tissue which has been the object of a very great number of morphological and physiological studies, has seldom been noted as a site of lymphocyte migration. It is usual, in fact, to stress the homogeneity of the cellular constitution of the epidermal layer. Nevertheless, studies on the lower vertebrates, even at a very early date (Ehrmann, 1885) showed the presence within this layer of mesodermal elements in the form of extensively branching pigment cells. Bolk and Göppert ('31) describe branched pigment cells and also cells probably leucocytic in nature as occurring in the epidermis of the majority of adult amphibia. In higher vertebrates, including man, cells differing from the ordinary epithelial cells have been described by various authors. The cells of Langerhans (Langerhans, 1868), are present chiefly in the more superficial portions close under the cornified layer and demonstrable by metallic impregnation techniques. They have been studied and identified as a distinct cell type by Bezecny ('34), Klein and Missriegler ('35) and Schaaf ('38). They show a cell body and a number of processes. They are considered as autochthonous elements of the epidermis, not as invaders, and as probably nervous elements.

Masson, however, ('21) considers the cells of Langerhans as beginning their development in the basal layer, gradually

being pushed toward the cornified layer, and as cells having the role of pigment distribution in the epidermis, and yet being of nervous origin. He considers another cell type, the clear cell, of which we shall have much to say in this paper, as related to the cells of Langerhans and like them, probably representing neuroglia of tactile nerves (Masson, '26).

These cell types are considered as ectodermal cells and as non-motile in nature. It is a noteworthy fact that almost no authors speak of mesodermal or motile cells in the normal epidermis of the higher vertebrates or of man. Thus Fraser ('28) in a detailed study of the development of the skin of the albino rat, which included not only fetuses and newborn animals but also postnatal stages up to 150 days, makes no mention of wandering cells in the epidermis. Nor does Hanson ('47) who studied specifically the histogenesis of the epidermis in the rat and mouse, carrying the observations up to animals of 100 days, mention any cells other than those of epithelial nature.

We have consulted also a number of papers dealing with experimental procedures and alterations of the epidermis under experimental conditions. Loeb, Haven and others ('30) reporting on changes in undernourishment in guinea-pig epidermis; Lasnitzki ('31), describing regeneration of epidermis of the guinea-pig at low atmospheric pressures; Gall, Lingley, and Hilcken ('41), observing the effects of x-rays on the skin of albino rats; and Williamson ('41) describing changes in the epidermis of rats on fat-free diets, fail to mention lymphocytes or other wandering cells in the epidermis of either experimental or control animals.

Several authors do point out, in special studies on the junction of epidermis and dermis, that the idea of a basement membrane, in the form of a barrier between the two layers, cannot be supported. Szordoray ('31) describes an interdigitation of the fine fibrillae of basal epithelial cells with the expanded ends of the lattice fibrils of the dermis.

Clerc ('41) finds fibrils ending freely with no barrier formation and an appearance of epithelial cells occasionally

breaking loose and descending into the dermis, probably to perish there under normal conditions.

The only publication which we have found that describes wandering cells in the normal epidermis of mammals is that by Kondo ('22). He found such cells, chiefly lymphocytes and histiocytes, in the epidermis of the rabbit and of man, occurring between the epithelial cells.

We have had the privilege of reading an unpublished manuscript by Andreasen. His observations, made in Cowdry's laboratory in 1947–48, concern primarily the presence of lymphocytes in the epidermis during methylcholanthrene carcinogenesis. He studied also, however, as controls, epidermis of 10 untreated mice of about 6 weeks of age. In all of them he found lymphocytes present, ranging in number from 2.0 to 3.8% of the total number of identifiable cells, with an average of 2.6%.

MATERIALS AND METHODS

The material used in this study consists of portions of the normal skin from the back and from the abdomen of 6 albino rats, each 300 days of age, and of normal human skin taken from the antecubital region, as biopsies, from 15 subjects. A very large amount of material from pedigreed rats from the Wistar Institute was available for a study on age changes in the skin. It was, in fact, the constant occurrence of lymphocytes in the epidermis of animals being studied in the age series that led us to consider it worth while to make an investigation of these cells as such.

We wish to express our deep appreciation to Doctor Edmund V. Cowdry for access to the excellent human biopsy material which is part of an extensive collection of material for a program of study on ageing of human skin, carried on by Evans, Cowdry and Nielson ('43) and Cowdry, Cooper and Smith ('47). The specimens were excised from volunteers, most of them feeble-minded but otherwise normal, under local anesthesia, employing a uniform surgical technique. All subjects were of the Caucasian race.

Both rat and human skin were fixed in Bouin's fluid, dehydrated, cleared and embedded in paraffin. Sections were cut generally at 5 μ. Several stains were employed for each specimen. For the rat tissue, hematoxylin and eosin, Heidenhain's iron-hematoxylin, and Mallory's triple stain were employed. For the human tissue, hematoxylin and eosin, Heidenhain's iron-hematoxylin, and Masson's trichrome stain were used.

After preliminary surveys of the sections stained by the various methods, a quantitative study involving the lymphocytes was undertaken for each specimen. This included the counting of 1000 nuclei (cells) from the stratum germinativum on the abdomen of each rat and the same number on the back of the animal. For the human material also, 1000 cells were counted for each subject. The study thus involves 12,000 cells of the rat epidermis and 15,000 of the human epidermis. The "counts," however, do not represent simply quantitative data. They involve the classification of certain of the cells as lymphocytes, of others as clear cells, and of still others as undetermined or intermediate cells. All counts were made under high magnification (oil immersion lens).

OBSERVATIONS

Lymphocytes were conspicuous in the epidermis in all of the rats studied. The epidermis is relatively thin in the rat and in general consisted in our sections of two to 4 layers of cells in the stratum germinativum. The basal cells seldom are cylindrical, generally appearing polyhedral, cuboidal or even elongated parallel to the surface. The large clear nucleus generally contains one or two conspicuous nucleoli. The cytoplasm is lightly stained with eosin and intercellular bridges are not prominent. In the iron-hematoxylin preparations epithelial fibrillae can be seen.

The human skin from the antecubital fossa shows an epidermis considerably thicker than that from the rat. The basal layer is usually composed of cylindrical or high cuboidal cells. Otherwise, however, the appearance of the epithelial

cells, lymphocytes, clear cells, and intermediate cells is very similar in the two species and the general description here given will serve for both.

The lymphocytes have deeply staining nuclei and about the nucleus is a "vacuole" or very clear area. This area we believe to represent the cytoplasm of the lymphocyte. The general appearance of the lymphocytes in the epidermis is similar to that seen in other locations where they are migrating into an epithelial layer, as in the trachea, or in the intestine when they have reached only to the bases of the epithelial cells.

Close observation shows that these cells have a marked effect on neighboring cells. It apparently is a mechanical pressure effect. Epithelial cells on either side and directly above the lymphocyte are seen to be distorted, their sides or bases sometimes greatly indented and the epithelial nucleus even may be altered in form. These changes seem to us to be due both to the migratory activity of the lymphocyte, and, after it has come to rest, to the great increase in volume in its cytoplasm, for the clear, vacuolar substance about the nucleus is many times wider than the cytoplasmic rim usually seen for lymphocytes not in an epithelial layer.

The lymphocytes are almost always in the basal layer, occasionally, especially where the epithelium is 4 or more cells in thickness, in the layer immediately above the basal one. It is a noteworthy fact that they are not found in the more superficial layers and we have not seen them in the stratum corneum.

The lymphocytes show no tendency to degenerate within the epidermis. We have not seen figures suggesting karyorrhexis or other type of nuclear destruction.

The only change which we feel that we have been able to follow, and which appears as a continuation of their initial change upon entering the epidermis, is a transformation into the "clear cell." Clear cells, "epithelial" cells with very lightly staining cytoplasm, rounded cell bodies, and nuclei usually somewhat darker than those of the regular epithelial

cells are abundant in the epidermis of the normal skin of man and of the rat. Like the lymphocytes, they occur almost always in the basal layer, and like the lymphocytes also, they appear to be exercising a pressure effect on adjoining epithelial cells. In fact, their resemblance to the lymphocytes is very great, so much so that we felt it impossible to say in every case whether a particular cell should be designated

TABLE 1

Data obtained by counts of 1,000 cells of stratum germinativum of epidermis of each albino rat in each region. All animals are 300 days of age. Nos. 156, 266 and 301 are males. Nos. 158, 222 and 303 are females

IDENTIFICATION OF ANIMAL		LYMPHOCYTES		INTERMEDIATE CELLS		CLEAR CELLS		MITOTIC FIGURES	
		no.	%	no.	%	no.	%	no.	%
156	Abdomen	18	1.8	4	0.4	45	4.5	1	0.1
	Back	48	4.8	1	0.1	105	10.5	1	0.1
158	Abdomen	17	1.7	3	0.3	83	8.3	0	0
	Back	24	2.4	11	1.1	76	7.6	3	0.3
222	Abdomen	29	2.9	15	1.5	39	3.9	0	0
	Back	40	4.0	9	0.9	85	8.5	0	0
266	Abdomen	20	2.0	12	1.2	89	8.9	0	0
	Back	40	4.0	7	0.7	87	8.7	0	0
301	Abdomen	9	0.9	10	1.0	24	2.4	1	0.1
	Back	25	2.5	3	0.3	80	8.0	0	0
303	Abdomen	16	1.6	9	0.9	58	5.8	1	0.1
	Back	25	2.5	7	0.7	75	7.5	0	0

as a lymphocyte or as a clear cell. Because of this circumstance we have formed a category known as "intermediate." Cells so designated resemble the lymphocyte on the one hand and the clear cell on the other hand and seem to form a perfectly graduated transition between the two types.

As seen in tables 1 and 2, the numbers of lymphocytes in normal epidermis are not inconsiderable and agree fairly well from individual to individual. Also, they are not markedly different when rat and man are compared. The num-

bers of clear cells are even larger and the three categories together make up a fair percentage of the total number of cells in the stratum germinativum.

For each specimen we have counted also the number of mitotic figures. There is very little mitosis present either in these adult rats or in the epidermis of the human subjects. Other possible ways of regeneration of the epithelium

TABLE 2

Data obtained by counts of 1,000 cells of stratum germinativum of epidermis from each of 15 human subjects, biopsy material from antecubital fossa

IDENTIFICATION NO.	AGE AND SEX	LYMPHOCYTES		INTERMEDIATE CELLS		CLEAR CELLS		MITOTIC FIGURES	
		no.	%	no.	%	no.	%	no.	%
A1	73 ♂	18	1.8	5	0.5	13	1.3	0	0
A2	30♂	27	2.7	8	0.8	15	1.5	0	0
A3	85 ♀	39	3.9	11	1.1	16	1.6	0	0
A4	75 ♀	32	3.2	7	0.7	25	2.5	0	0
A5	82 ♀	33	3.3	14	1.4	27	2.7	1	0.1
A6	62 ♀	14	1.4	8	0.8	24	2.4	0	0
A7	81 ♀	33	3.3	12	1.2	46	4.6	0	0
A8	19 ♂	14	1.4	15	1.5	33	3.3	0	0
A9	54 ♂	22	2.2	23	2.3	21	2.1	0	0
A20	15 ♂	36	3.6	13	1.3	24	2.4	0	0
A21	20 ♂	30	3.0	14	1.4	47	4.7	0	0
A22	20 ♂	20	2.0	14	1.4	38	3.8	0	0
A23	24 ♂	26	2.6	7	0.7	20	2.0	0	0
A24	24 ♂	20	2.0	16	1.6	19	1.9	1	0.1
A25	20 ♂	18	1.8	19	1.9	22	2.2	0	0

include the process of amitotic division. We looked for evidence of amitosis with negative results, with the possible exception of some figures in the spinous layer in some of the senile human subjects.

The considerable numbers of lymphocytes, the intermediate cells which appear as transitions from lymphocytes to clear cells, and the many clear cells, which seem definitely epithelial, have seemed to us to indicate some very important role for the migrating lymphocytes in the economy of the epidermis. The paucity of mitotic figures, and the lack

of amitosis, together with the need for constant renewal of cells, have led us to consider this role to be a regenerative one, a process of differentiation of lymphocytes into epidermal cells.

DISCUSSION

The impression that the mitotic divisions present in epidermis are not numerous enough to account for the amount of cell renewal necessary has been expressed by various authors. Patzelt ('28, p. 664) says of such mitotic division: "Since this occurs much too seldom when compared with the great amount of desquamation, the necessary cell replacement may take place in large part at the upper boundary of this layer through direct cell division, which occurs here instead of mitosis, due to the poor nutritive conditions."

It is true that we are unable to state the rate of desquamation in terms of actual numbers of cells lost. Voit ('30) in a careful chemical study has determined that the weight of desquamated cells from the epidermis, exclusive of hairs, is about 116.14 grams per square meter of surface per year. While this is of interest, it does not at present permit us to calculate numbers of cells lost. Investigators who have had much to do with the subject, however, as Patzelt, quoted above, have felt that a discrepancy exists between the concept of relatively rapid renewal and the small numbers of mitotic figures present.

Thuringer ('28, p. 1) says: "The fact that in normal epidermis insufficient mitotic figures are encountered to compensate for the constant loss of cells from the surface, and the relative frequency with which binucleated cells may be observed, especially near the stratum granulosum, has strengthened the assumption that amitosis perhaps takes place along with mitosis." Some of the extremely low figures which Thuringer cites for mitosis in adult human epidermis from an executed subject and with "instantaneous" fixation are of interest. For the ear the figure is 1:268,275; for the leg (anterior tibial region) 1:378,325. For the scalp it is many times more common, the figure 1:2,414 being given.

In a study of the response of palmar and plantar epidermis to stimulation in the cat (Thuringer, '39) the number of mitoses in a control specimen was 78:65,018 or little more than one per thousand.

While the variations in numbers of mitoses are considerable when different regions are compared, these figures seem to us to be surprisingly low if mitosis is the only, or even the major method of supplying new cells. With the figure given for the anterior tibial region, for example, and allowing two hours per mitosis (W. Lewis and M. R. Lewis, '17) an individual cell would seem to have an "intermitotic period" stretching into years.

Our own figures on numbers of mitoses are very low (tables 1 and 2). It certainly can be argued that the "zero" figure per 1,000 cells does not give an actual proportion, yet the fact that counting 1,000 cells of the stratum germinativum yielded no mitotic figures in so many cases both in rat and man indicates the infrequency of the process.

Blumenfeld ('42) gives figures for the number of mitoses in the submaxillary gland, renal cortex and epidermis of albino rats about 28 days of age. His maximum count is approximately 75 mitotic figures per 1,000 fields. This figure, considering the number of cells per field (60, according to him, in the submaxillary gland) would be a little over one per thousand. He found a rhythmic curve for mitosis in the epidermis but his lowest figures were from 6–12 P.M., at a time when none of our animals was sacrificed.

It is interesting that his numbers of mitotic figures for epidermis are little different than those for renal cortex and submaxillary gland where the mitoses are simply an accompaniment of the growth of the organ.

Cameron ('36), in vertebrates of a totally different class, the Amphibia, was struck by the very small numbers of mitotic figures in older tadpoles and in adult frogs. He also studied 24-hour series of mounts of fresh skin and found no evidence for "bursts" of mitosis occurring in rhythmical

fashion which might explain the infrequency of mitosis in the sections.

If mitosis is not the only, nor the major source of new cells, at least two other possible sources can be thought of as possibilities. The first of these is amitosis. The second is the addition to the epidermis of non-epidermal cells with subsequent differentiation of such cells to become epithelial in character.

Search for amitotic division in our material has given almost uniformly negative results. In the rat we have seen none, in some of the oldest human subjects there are a few suggestive figures. Ortiz-Picon ('34) found no amitosis in the epidermis of the mouse.

The second possibility meets with a considerable, although we believe largely theoretical, obstacle. In order to be added to the epidermis, cells would have to come from tissues of different germ-layer origin, and logically from the mesoderm. Recent work on experimental embryology, it is true, is doing much to alter ideas on the absolute specificity of the germ layers. Holtfreter ('47, p. 58) says "The ectoderm cells from an amphibian gastrula are capable of differentiating into almost any kind of cell type," and again (p. 89) "Neuroblasts, mesectoderm cells and leucocytes start out from the same architectural plan."

We have found in the literature two authors who have made the definite suggestion that the epidermis is renewed by the addition of cells from the underlying mesodermal tissue.

Cameron ('36), cited above, felt that both in normal and x-rayed frog-skin, "spindle-shaped" mesodermal cells made their way into the epidermis and were incorporated into it, becoming epithelial. Subjecting the animals to x-rays decreased the already low numbers of mitotic figures, yet the epidermis did not change in thickness, but an increase in the mesodermal cells beneath the epidermis and passing into it was seen.

MacCardle ('41) emphasized the clear cells of the epidermis, particularly in neurodermatitic lesions, where these cells are numerous, large and conspicuous. He states that such cells are present not only in the lower layers of the epidermis but also as isolated or wandering cells in the dermis. He says (p. 27) "It seems that in neurodermatitis the clear cells are more frequently related to regeneration of new epidermis than to any other physiologic phenomenon, for in recently healed lesions that show large numbers of faintly stained mitotically dividing basal and spinous cells, the clear cells invading the basal layer are unusally numerous, . . . This relation to regeneration is far more consistently uniform than is the relation of clear cells to pigmentation."

In our investigations we have felt that the recognition of the cell type as it first enters the epidermis, in the normal state, is of considerable importance. Our previous studies on lymphocytes in epithelium of the intestine (Andrew and Andrew, '45; Andrew and Collings, '46; Andrew and Sosa, '47) and of the trachea (Andrew and Burns, '47) have given an opportunity to become acquainted with the appearance of these cells, and their characteristics in epidermis do not differ essentially from those seen in other epithelia for intercellular lymphocytes. The nucleus is dark, with heavy granules of chromatin, but it is not pycnotic. The cytoplasm at first appears as a bright "vacuole-like" structure, probably at the time of a rapid increase in volume.

The transition from lymphocyte to clear cell is marked by a gradual differentiation of the nucleus, particularly a lightening in staining qualities, and by a further increase in size both of cytoplasmic body and nucleus. From clear cell to ordinary epithelial cell, the process seems to be one primarily of cytoplasmic differentiation, the cell body acquiring a greater degree of stainability and developing the epithelial fibrillae.

While we emphasize here the small number of mitotic figures in normal epidermis, we are, of course, aware of the

fact that under certain conditions, such as hormonal stimulation or the action of carcinogens, the number increases greatly. An interesting field for speculation and observation may be opened by the fact that some at least of the mitotic figures which we have seen in normal epidermis seem to be those of clear cells or even lymphocytes.

We also are presenting no evidence against a rhythmicity in occurrence of mitotic division. In relation to "bursts" of mitotic activity, we have seen none of any magnitude in our material. In one rat two mitotic figures were found side by side. These we consider very possibly to be occurring in daughter cells formed by a previous division.

Billingham ('48 and '49) has stressed the presence in human skin, both white and colored, of two races of cells, the "more or less rounded Malpighian cells" and the dendritic cells.

We believe that the lymphocytes, clear cells, and ordinary epithelial cells are members of the first group.

The multipotential characteristics of lymphocytes, the ability which they possess to develop into any one of a number of cell types, has become increasingly evident. Studies on sections and on tissue cultures have shown that the small lymphocyte may develop into polymorphonuclear granulocytes (Bloom, '37; Kingsbury, '44) macrophages (Maximow, '09) and even fibroblasts (Bloom, '28).

The epithelial cells scarcely can be thought to be more different from, say, the fibroblast than is the latter from the small lymphocyte, and the conception of the differentiation of lymphocytes to epithelial cells does not seem so extraordinary when we consider the facts already learned in regard to their multipotentiality. We have already suggested this possibility for intestinal epithelium (Andrew, '48). In the epidermis the process appears relatively easy to follow and the sequence to be from small lymphocyte through clear cell to the recognized epithelial type.

SUMMARY AND CONCLUSIONS

1. Lymphocytes are of constant occurrence in the normal epidermis of the rat and of man. They constitute about 1–4% of the cells of the stratum germinativum.

2. The lymphocytes are located primarily in the basal layer. They are not found in the superficial layers of the stratum germinativum.

3. There are no indications of degeneration of lymphocytes within the epidermis.

4. The lymphocytes acquire a bright, "vacuole-like" body. The nucleus differentiates, becoming lighter staining.

5. The cell increases in size, becoming recognizable as a "clear cell." By differentiation of the cytoplasm, the clear cell becomes an ordinary epithelial cell.

6. The process of constant migration of lymphocytes into epidermis and their differentiation there to epidermal cells can account for the relatively low numbers of mitotic figures seen and for the apparent lack of any other mode of rapid regeneration.

LITERATURE CITED

ANDREASEN, E. 1948 Intraepidermal lymphocytes in carcinogenesis induced in mice by methylcholanthrene (unpublished manuscript).

ANDREW, W. 1948 Peculiarities of nuclear division in the crypts of Lieberkühn with evidence for the occurrence of differentiating mitosis. Anat. Rec., *101:* 673–674.

———— 1949 The role of lymphocytes in the normal epidermis. Anat. Rec., *103:* 419.

ANDREW, W., AND N. V. ANDREW 1945 Mitotic division and degeneration of lymphocytes within the cells of intestinal epithelium in the mouse. Anat. Rec., *93:* 251–277.

ANDREW, W., AND M. R. BURNS 1947 Leucocytes in the tracheal epithelium of the mouse. J. Morph., *81:* 317–341.

ANDREW, W., AND C. K. COLLINGS 1946 Lymphocytes within the cells of intestinal epithelium in man. Anat. Rec., *96:* 445–458.

ANDREW, W., AND J. M. SOSA 1947 Mitotic division and degeneration of lymphocytes within cells of intestinal epithelium in young and in adult white mice. Anat. Rec., *97:* 63–98.

BEZECNY, R. 1934 Die Langerhanssche Zelle. Arch. f. Dermat. u. Syph., *169:* 544–561.

BILLINGHAM, R. E. 1948 Dendritic cells. J. Anat., *82:* 93–109.

———— 1949 Dendritic cells in pigmented human skin. J. Anat., *83:* 109–115.

BLOOM, W. 1928 Mammalian lymph in tissue culture from lymphocyte to fibroblast. Arch. f. exp. Zellforsch., *5:* 269–307.

———— 1937 Transformation of lymphocytes into granulocytes in vitro. Anat. Rec., *69:* 99–121.

BLUMENFELD, C. M. 1942 Comparison of periodic mitotic activity in epidermis, renal cortex and submaxillary salivary gland of the albino rat. Arch. Path., vol. 33, pp. 770–776.

BOLK, L., AND E. GÖPPERT 1931 Handbuch der vergleichenden Anatomie, I, p. 325. Urban and Schwarzenberg, Berlin and Vienna.

CAMERON, J. H. 1936 The origin of new epidermal cells in the skin of normal and x-rayed frogs. J. Morph., *59:* 327–350.

CLERC, O. W. 1941 Uber die Verbindung des Epithels mit dem darunter gelegenen Gewebe in der Haut. Arch. f. path. Anat., *307:* 370–379.

COWDRY, E. V., Z. COOPER AND W. SMITH 1947 Program of research on aging of the skin. J. Geront., *2:* 31–44.

EHRMANN, S. 1885 Untersuchungen über die Physiologie und Pathologie des Hautpigmentes. Arch. f. Dermat. u. Syph., *17:* 507–532.

EVANS, R., E. V. COWDRY AND P. E. NIELSON 1943 Ageing of human skin, influence of dermal shrinkage on appearance of epidermis in young and old fixed tissues. Anat. Rec., *86:* 545–565.

FRASER, D. A. 1928 The development of the skin of the back of the albino rat until the eruption of the first hairs. Anat. Rec., *38:* 203–224.

GALL, E. A., J. R. LINGLEY AND J. A. HILCKEN 1941 Comparative experimental studies of 200 kilovolt and 1000 kilovolt roentgen rays; III. The biologic effect on the skin of the albino rat. Am. J. Path., *17:* 319–334.

HANSON, J. 1947 The histogenesis of the epidermis in the rat and mouse. J. Anat., *81:* 174–197.

HOLTFRETER, J. 1947 Changes of structure and the kinetics of differentiating embryonic cells. J. Morph., *80:* 57–93.

KINGSBURY, B. F. 1944 Tonsillar epithelium and the lymphocyte: an examination of the palatine tonsil of the cat. Am. J. Anat., *75:* 233–261.

KLEIN, B. M., AND A. MISSRIEGLER 1935 Langerhanssche Zellen in gut und bösartigen Neoplasmen. Arch. f. Dermat. u. Syph., *171:* 280–300.

KONDO, K. 1925 (1922) Studien über die Wanderzellen in der Haut. I. Mitt. Über die Wanderzellen in der Epidermisschicht von Menschen und Kaninchen. Jap. Jour. med. Sciences, *2:* 59–60 (abstracted in German from the Japanese in Kyoto Ig. Z., *19:* 386–407).

LANGERHANS, P. 1868 Über die Nerven der menschlichen Haut. Virch. Arch., *44:* 325–337.

LASNITZKI 1931 Über die Regeneration des Hautepithels bei vermindertem Luftdruck. Virch. Arch., *281:* 66–72.

LEWIS, W. H., AND M. R. LEWIS 1917 The duration of the various phases of mitosis in the mesenchyme cells of tissue cultures. Anat. Rec., *13:* 359–368.

LOEB, L., AND F. L. HAVEN 1929 The relation between functional states of the sex organs in the female guinea pig and the proliferation in the epidermis. Anat. Rec., *43:* 1–26.

LOEB, L., F. L. HAVEN, J. T. GENTHER AND H. FRIEDMAN 1930 Effect of undernourishment on proliferative activity and structure of epidermis of guinea-pig. Anat. Rec., *46:* 55–64.

MACCARDLE, R. C., M. F. ENGMAN, JR., AND M. F. ENGMAN, SR. Histology of neurodermatitis. From Studies on Neurodermatis, American Medical Association, Copyright 1941, 1942, 1943, 1–29.

MASSON, P. 1921 Les cellules de Langerhans: leur rôle dans les échanges dermato-épidermiques. Bull. Soc. franc. de dermat. et syph. (Reunion dermat. Strasbourg), *28:* 7–13.

———— 1926 Les naevi pigmentaires, tumeurs nerveuses. Ann. d'anat. path., *3:* 417–453.

MAXIMOW, A. 1909 Untersuchungen über Blut und Bindegewebe. I. Die frühesten Entwicklungsstadien der Blut und Bindegewebeszellen beim Säugetierembryo, bis zum Anfang der Blutbildung in der Leber. Arch. f. mikr. Anat., *73:* 444–461.

MAXIMOW, A., AND W. BLOOM 1947 Textbook of Histology. W. B. Saunders & Co., Philadelphia.

ORITZ-PICON, J. M. 1934 Über Zellteilungsfrequenz und Zellteilungsrhythmus in der Epidermis der Maus. Ztschr. f. Zellforsch u. mikr. Anat., *19:* 488–509.

PATZELT, V. 1928 Bau und Verhornung der menschlichen Epidermis. Wien med. Wchnschr., *78:* 663–665.

SCHAAF, F. 1938 Eine Methode zur sicheren Darstellung der Langerhansschen Zellen in der Epidermis des Menschen, in Meerschweinchen und Katzenpfote. Arch. f. Dermat. u. Syph., *176:* 535–543.

SCHAFFER, J. 1927 Leucocyten im Epithel, in W. v. Mollendorff's Handbuch der mikroskopischen Anatomie des Mensche, II/1, Die Gewebe, *1:* 91–96.

SZORDORAY, L. 1931 The structure of the junction of the epidermis and the corium. Arch. Dermat. and Syph., *23:* 920–925.

THURINGER, J. M. 1928 Studies on cell division in the human epidermis. II. A. Rate of cell division in the prepuce. B. Influence of various factors on cell division. Anat. Rec., *40:* 1–13.

———— 1939 The mitotic index of the palmar and plantar epidermis in response to stimulation. J. Invest. Dermat., *2:* 313–326.

WILLIAMSON, R. 1941 A note on the epidermis of the rat on a fat-free diet. Biochem. J., *35:* 1003–1005.

VOIT, E. 1930 Uber die Grösse der Erneuerung der Horngebilde beim Menschen III. Mitteilung. Die Oberhaut. Ztschr. f. Biol., *90:* 549–553.

PLATES

PLATE 1

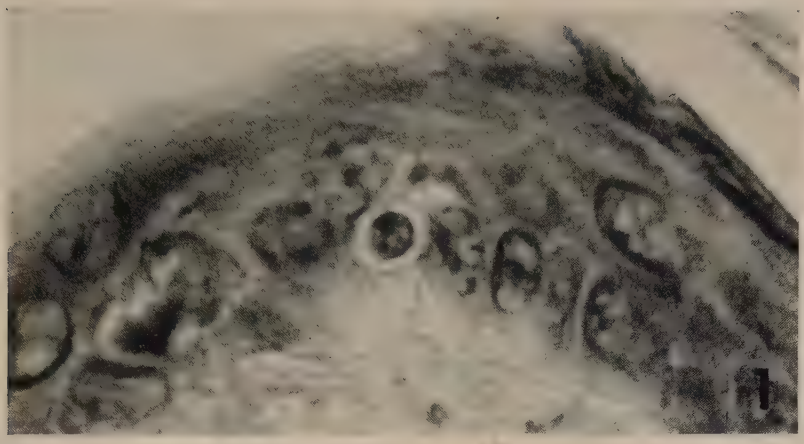
EXPLANATION OF FIGURES

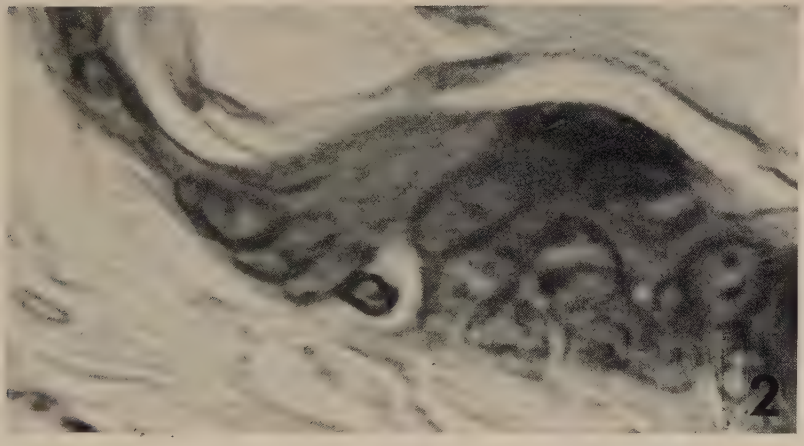
The magnification of all figures in plates 1–4 is × 1600. Figures 1–9 are from abdominal skin of 300 day old female albino rats stained with Harris' hematoxylin and eosin.

1 A lymphocyte in the epidermis. This cell differs from lymphocytes in the underlying dermis only in the clear "vacuole-like" cytoplasm.

2 A cell of a type intermediate between the lymphocyte, above, and the clear cell, below. The nucleus is assuming epithelial characteristics, the cytoplasm remains with practically no stain.

3 A clear cell. The nucleus is very similar to that of the epithelial cell on the right. The cytoplasm is very lightly stained.


1


2

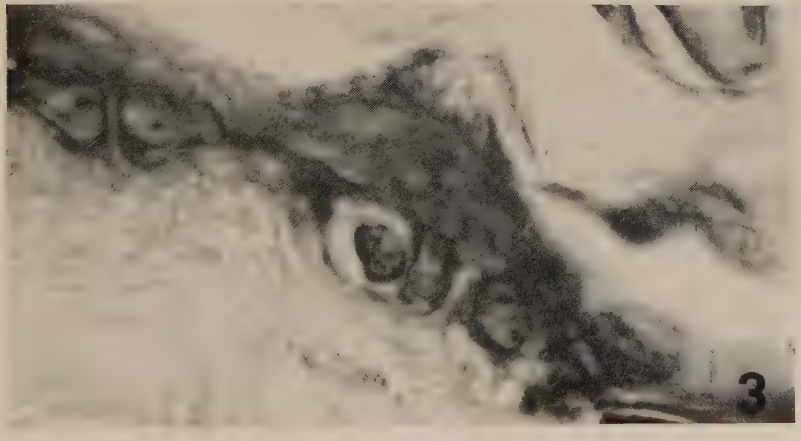

3

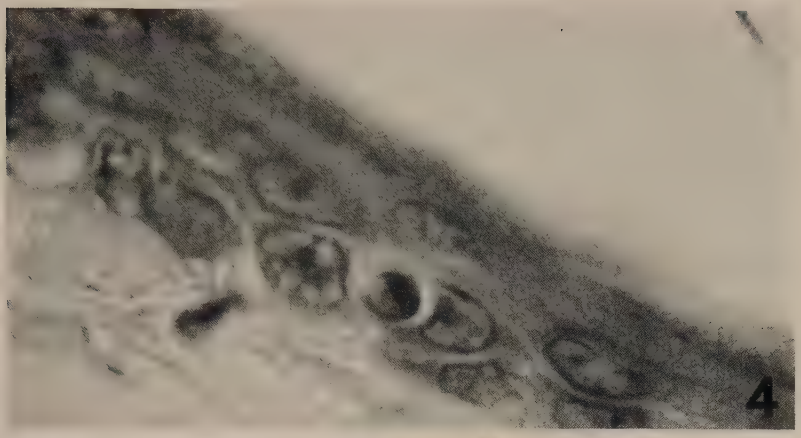
PLATE 2

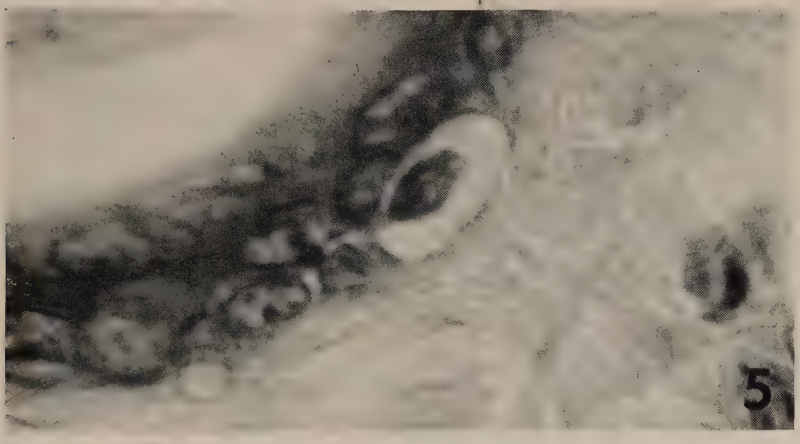
EXPLANATION OF FIGURES

4 A lymphocyte in the basal layer of the epidermis. Here the cell outline is well seen and the pressure due to increase in size apparently is evidenced by the deformation of the epithelial cell to the right.

5 A large clear cell. Here the nucleus in form and appearance is almost the same as that of the epithelial cells, and the orientation of the cell conforms to that of the nearby cells.

6 A cell which retains some clear areas in the cytoplasm but which varies little from the ordinary epithelial cells.


4


5

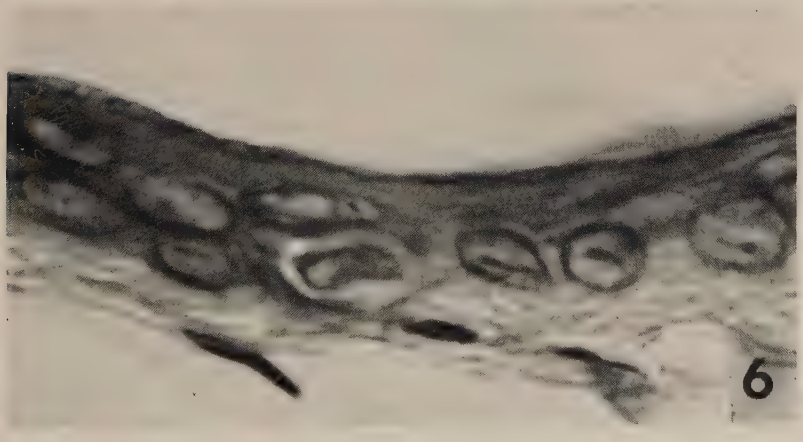

6

PLATE 3

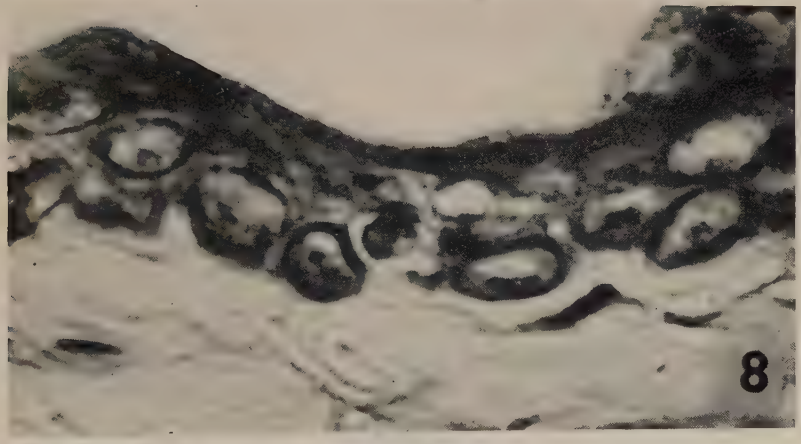
EXPLANATION OF FIGURES

7 A lymphocyte in the epidermis. This again illustrates the very clear cytoplasm of lymphocytes in the epithelium and the tendency to deform adjoining epithelial cells by increase in size of the cell body of the wandering cell.

8 A cell of intermediate type showing pressure effect on adjoining epithelial cells.

9 A clear cell which in size and shape of nucleus and cell body much resembles a lymphocyte. The interior of the nucleus has become light, however.

7


8

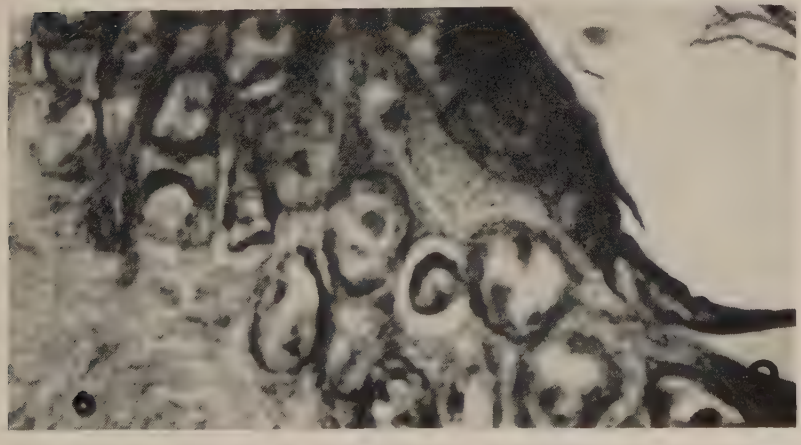


PLATE 4

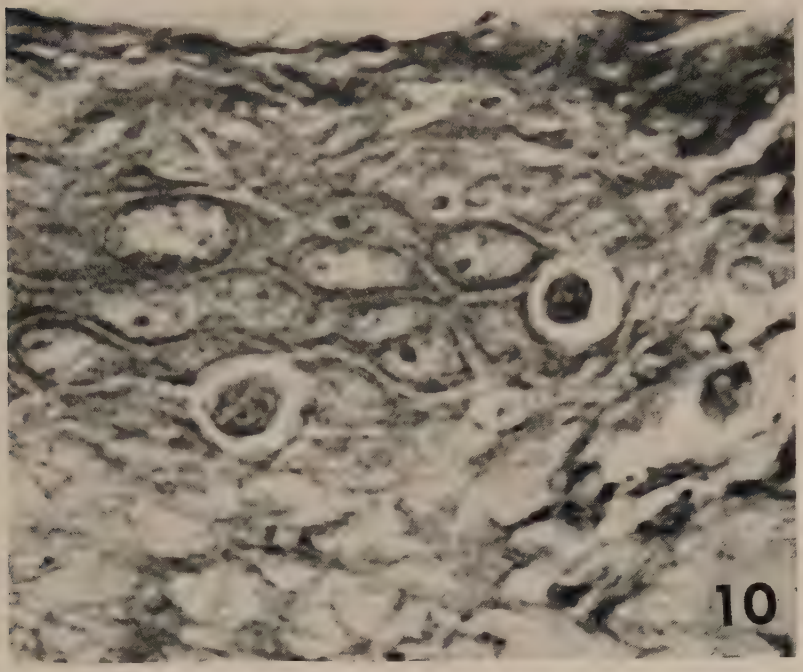
EXPLANATION OF FIGURES

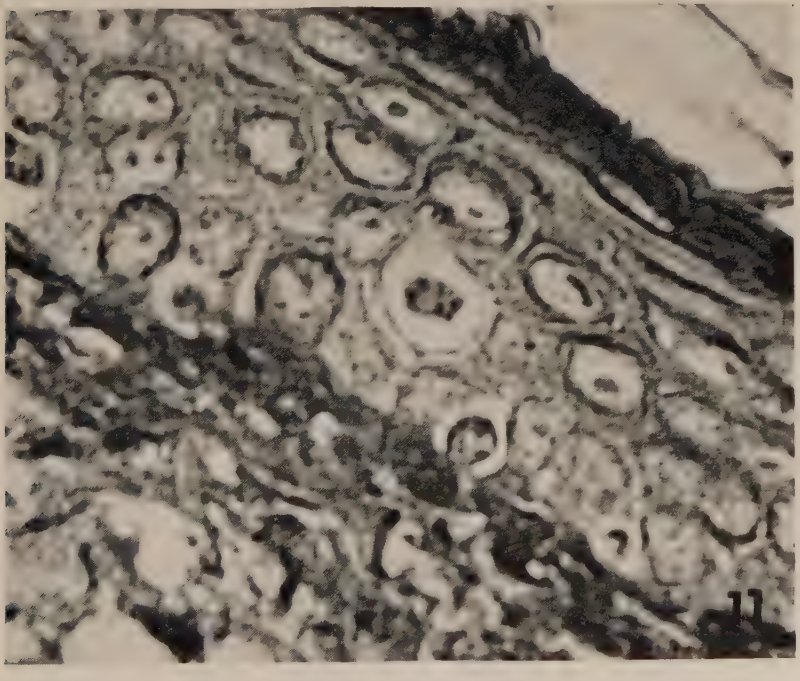
Human epidermis

Figures 10 and 11 are from a skin biopsy from the antecubital fossa of a 20 year old white male subject. Masson's trichrome stain.

10 A cell with a definitely lymphocytoid nucleus on the right and with a large clear cytoplasmic body. On the left a typical clear cell.

11 In the basal layer a clear cell with a relatively small cell body and in the spinous cell layer a larger clear cell.


10


11

ANISOTROPIC LIPIDS IN THE SEBACEOUS GLANDS OF THE RABBIT[1]

WILLIAM MONTAGNA
Arnold Laboratory, Brown University, Providence 12, Rhode Island

SIX FIGURES

INTRODUCTION

The sebaceous glands of the rabbit contain accumulations of anisotropic lipids only in the periphery of the acini. This distribution of anisotropic lipids is not like that found in the glands of the rat, mouse, hamster, dog, and cat, where the birefringence is found in the sebum and in the mature and degenerating sebaceous cells, but not in the peripheral cells. The properties and characterization of these lipids in the rabbit have been studied by the use of Sudans and other oil-soluble substances, and by some cytochemical tests. The peripheral anisotropic lipids represent, at least in part, cholesterol esters. The presence of these lipids in the periphery of sebaceous glands is undoubtedly characteristic of the rabbit. The striking species difference should caution one against drawing too broad generalizations in the study of these glands from one animal form only.

MATERIAL AND METHODS

The sebaceous glands were studied from the skin of the external auditory meatus of the rabbit. This region was chosen in preference to others because the glands therein are numerous and active. These glands are sufficiently like

[1] This work was supported by a grant from the United States Public Health Service, R. G. 1036, administered by Dr. J. B. Hamilton, Long Island College of Medicine.

those in the body skin (flank, dorsum, and belly) to be considered typical.

Specimens collected from more than 20 adult males were fixed in: (a) 10% formalin with or without a suspension of calcium carbonate or magnesium carbonate; (b) formal-calcium of Baker ('44); (c) buffered 10% formalin (Lillie, '48). After a fixation of 24 hours to three months, frozen sections were cut 5 μ to 10 μ thick. For the study of birefringence, sections were mounted unstained in glycerin, or glycerin jelly, or in a 2% aqueous solution of levulose. To reveal saponifiable lipids, sections were mounted in Molisch fluid (from McClung, '37) and maintained in a moist chamber for 5 to 10 days. For secondary osmiophilia, sections were placed in 2% osmic acid for 10 minutes to 5 days at 37°C. and then washed in 70% alcohol (see Hoerr, '36). Sections treated with Sudan IV, Sudan black, oil-blue N, oil-brown N, and oil-red O (Lillie, '48) were studied under bright light and under polarized light. For the demonstration of unsaturated neutral lipids (triglycerides), sections were treated with the Nile-blue sulfate method of Cain ('47); for phospholipids, Baker's ('46) acid hematein test; for cholesterol and cholesterol esters, application of modified Liebermann-Burchard test (Kay and Whitehead, '37; Lison, '36); sections were also treated with 0.5% digitonin in 50% alcohol. Other manipulations of these tests are described in the observations.

Frozen sections of fresh unfixed tissues were studied under polarized light and compared with fixed material.

I wish to thank my assistant, Miss Patricia Kenyon, for her excellent help in the preparation of this material.

OBSERVATIONS

In freshly fixed frozen sections studied under polarized light, the sebum and the degenerating and mature sebaceous cells show scattered anisotropic granules and rodlets. The peripheral sebaceous cells, varying from one to three or more

cells in thickness, however, are usually loaded with anisotropic spherocrystals (fig. 1). In unfixed frozen sections the anisotropy is essentially similar to that in fixed material, with the exception that the spherocrystals are not seen as clearly as in fixed material.

The use of different fixatives, the length of fixation of tissues, the length of time that sections have been sectioned before they are studied under polarized light, and alterations in temperature, all exert some influence upon the general appearance of the birefringence.

It is inadvisable to fix tissues for longer than one or two days. Most fixatives after a time alter to some extent the birefringence of lipids. Fat cells, for instance, which in freshly-fixed material are never birefringent, in tissues fixed for one week or longer often develop rosettes of strongly birefringent acicular crystals. Study of such preparations, then, can be misleading. Since formaldehyde solutions are acid, the addition of a suspension of magnesium carbonate or calcium carbonate, brings the solution to neutrality. After tissues have been stored in them for a week or longer, however, the solutions become acid again. After tissues are stored in formal-calcium for three weeks, the solution may attain a pH of 4.5. The most satisfactory fixative is 10% formaldehyde buffered to pH 7.2. This solution is stable, and tissues stored in it show little alteration in anisotropy.

In sections of tissues fixed for longer than one week in 10% formalin, with or without a suspension of calcium carbonate, or in formal-calcium, long acicular crystals are found among spherocrystals at the periphery of the sebaceous glands. After a few hours there is a sharp increase in the number of acicular crystals in many glands. These may become so numerous in some acini that they mask the presence of spherules. This phenomenon occurs less frequently in sections of tissues fixed in buffered formalin.

Heating sections to 60°C. for one minute destroys temporarily all birefringence. It reappears virtually unchanged within a few minutes as the sections become cooled to room

temperature. When sections are cooled to —2°C., the sebum and all sebaceous lipids become brightly anisotropic. The induced anisotropy does not appear to be crystalline, and vanishes after one or two minutes, as the sections become rewarmed to room temperature.

In sections mounted in Molisch fluid (from McClung, '37) for three to 5 days, the birefringent crystals at the periphery of sebaceous glands remain unchanged, but there is an increase in birefringence in the sebum and in the mature and degenerating sebaceous cells. Should sections be maintained in Molisch fluid for a longer time, the secondary birefringence disappears. This intermittent birefringence probably represents partially saponified lipids.

The birefringence at the periphery of the glands is not appreciably altered when sections are placed in 50%, 70%, and 80% alcohol for 10 hours or longer at room temperature. The spherocrystals, but not the acicular crystals, are abolished by 90% alcohol; 95% alcohol or acetone destroy all lipid birefringence.

Treatment of sections for two hours or longer with 0.5% alcoholic solution of digitonin, either at room temperature or at 37°C., shows no visible change in the birefringence. Subsequent immersion of these sections in 95% alcohol, or in acetone, or in ether, destroys all birefringence, indicating that there are no insoluble digitonides.

In frozen sections placed in 2% osmic acid at 37°C. for 10 minutes to 5 days, and subsequently placed in 70% alcohol, only the sebum is appreciably blackened. All other sebaceous elements become a murky gray or brown color. The lipid droplets in the mature sebaceous cells are osmiophobic even though they appear to contain unsaturated neutral lipids (vide infra), which in adipose tissue become blackened. The birefringent lipids in the peripheral cells are weakly osmiophilic. This is the only recognizable osmiophilia in the sebaceous cells and it is not intensified by longer treatment with osmic acid. In osmicated sections the birefringence of

the peripheral cells is lost, but that in the darkened sebum is intensified.

When sections are treated for 5 to 10 minutes with Sudan IV, the sebum and the degenerating and mature sebaceous cells become a brilliant red, but the periphery of most glands is a pale orange color, or it remains colorless (fig. 2). Under polarized light the poorly stained peripheral areas of the glands show birefringent lipids (fig. 3). The spherocrystals may appear pale pink, but the acicular crystals remain colorless.

After 10 minutes in oil-blue N only a few acicular crystals are still birefringent at the periphery of the glands. With shorter treatment (two minutes), the normal anisotropicity is unchanged and the crystals are a delicate blue. This oil-soluble substance, then, has greater over-all affinity for lipids than Sudan IV.

Oil-red O and oil-brown N give results which resemble those obtained with Sudan IV.

When sections are treated for one minute with Sudan black, the sebum and the mature sebaceous cells are a deep blue-black, and the peripheral cells contain numerous blue granules. Under polarized light (fig. 5) the peripheral spherocrystals are pink, but the acicular crystals are either colorless or light blue. If sections are treated for 10 minutes, the birefringence is practically abolished although a few pink spherocrystals may still be present at the periphery of the glands.

When treated with Nile-blue sulfate, the sebum and the lipids in the degenerating and mature sebaceous cells become a bright rose color (indicating unsaturated neutral lipids), but the peripheral cells are usually blue. Intermingled with the blue peripheral color there may be small pink globules which give these areas a lilac or purplish color. Under polarized light, the peripheral birefringence is localized entirely in the blue-colored peripheral cells (fig. 4). Neither form of crystals is colored by Nile-blue, although

the crystals are so brilliant that the blue or pink in their vicinity imparts some color to them.

In sections treated with the acid hematein test for phospholipids, a deep blue, positive color is found in the sebum and in the degenerating sebaceous cells. Fine black granules are found in the cytoplasm of nearly all the sebaceous cells, but the lipid droplets remain unstained. The birefringent crystals remain unstained and unchanged.

After the application of modifications of the Liebermann-Burchard test to frozen sections, the sebum and all of the sebaceous cells attain a variety of intense colors which gradually turn to a positive deep blue-green. The substance responsible for this reaction (cholesterol or cholesterol esters) is present in the peripheral birefringent lipids as well as in the isotropic lipids of the sebaceous glands.

DISCUSSION

The characteristic anisotropy of lipid substances, taken by itself, is hardly more specific than their affinity for Sudan and other oil-soluble substances. Even when birefringent crystals are in the form of spherules, it is not possible to identify them with certainty, since nearly all lipids, with the possible exception of fatty acids, may be in this form. However, with the proper manipulations, it is possible to attain some characterization of these birefringent substances.

The sebaceous glands of the rabbit show birefringent crystals in the periphery of the glands, while the sebum and other sebaceous cells which are laden with lipids are nearly isotropic. This is unlike the situation in the glands of other animals (rat, Montagna and Noback, '47; dog, Montagna and Parks, '48; cat, Montagna, '49; hamster, Montagna and Hamilton, '49) where birefringence is always found in the sebum and in the mature sebaceous cells (fig. 6).

Although the peripheral birefringent crystals in the sebaceous glands of the rabbit are usually in the form of spherules, depending upon the kind and length of fixation, some glands may acquire secondarily numerous large acicular

crystals. These may become so numerous in some acini that they mask the spherocrystals already there. The long crystals resemble those which form in adipose tissue after partial saponification. They resist coloration with practically all the oil-soluble substances used. Perhaps they are crystals of fatty acids.

The peripheral anisotropic crystals have very little affinity for Sudan IV and remain brilliantly anisotropic even after prolonged treatment in this substance. Treatment with Sudan black for one to two minutes renders the spherocrystals a rose color, while the acicular crystals usually remain colorless. Longer treatment with Sudan black renders them isotropic. Nile-blue sulfate reveals unsaturated neutral lipids (stained a rose color) in the sebum, and in the degenerating and mature sebaceous cells. Rose-colored lipids are nearly absent from the periphery of the glands, which are colored blue. The birefringent crystals which are present only in the blue-colored region of the glands, are unaffected by Nile-blue.

Assuming that the acid hematein test is really specific for lipine, the birefringent elements cannot be phospholipids since they remain unstained.

The application of the Liebermann-Burchard test to sebaceous glands shows a positive green reaction throughout the gland, including the peripheral birefringent zone. Since insoluble digitonides are not formed in the gland, positive reaction of the Liebermann-Burchard test may demonstrate cholesterol esters.

The only positive reaction given by the birefringent lipids is that of the Liebermann-Burchard test. These lipids then, may represent cholesterol esters. Since cholesterol esters are also revealed in the rest of the gland, one wonders why only the peripheral lipids are highly birefringent. The findings of Okey ('44), that high concentrations of cholesterol in the liver are not birefringent when combined with fat, may throw some light upon this problem. In the periphery of sebaceous glands there appears to be little admixture of unsaturated

neutral lipids with Liebermann-Burchard positive substances. In the sebum and in the mature sebaceous cells these lipids are abundant and together, and birefringence is minimal. Furthermore, those acini whose peripheral cells are pink after Nile-blue, show a negligible peripheral birefringence. It is not possible to state why the birefringence in the sebaceous glands of the rabbit should be different from that of other animals, as for instance, the cat (fig. 6). In these other animals anisotropic lipids are abundant in the sebum and in the mature sebaceous cells where unsaturated neutral lipids and positive Liebermann-Burchard lipids are found together. Factors other than those stated above then, must be responsible for these discrepancies.

SUMMARY

In the sebaceous glands of the rabbit, abundant birefringent lipids are found only at the periphery of the acini, while the sebum, the degenerating, and the mature sebaceous cells are nearly isotropic. Birefringent lipids are principally in the form of spherules, but in some glands there are also numerous acicular crystals. Acicular crystals are more abundant in sections of tissues stored in fixatives for one week or longer. In tissues fixed in buffered 10% formalin, acicular crystals are less numerous.

The anisotropic lipids are not dissolved by 50%, 70%, and 80% alcohols at room temperature. In 90% alcohol the spherocrystals are abolished but the acicular crystals are not. In 95% alcohol or in acetone all anisotropicity is abolished. These crystals have little affinity for Sudan IV, oil-red O, or oil-brown N. Oil-blue N colors them. After short treatment with Sudan black the spherules are colored pink, and the acicular crystals are weakly colored blue. Longer treatment colors them intensely blue-black and renders them isotropic. Birefringent lipids are not colored by Nile-blue sulfate. After the Liebermann-Burchard test the anisotropic lipids, as well as the other glandular elements, give a positive

green color. The spherocrystals may represent cholesterol esters; the acicular crystals, fatty acids.

LITERATURE CITED

BAKER, J. R. 1944 The structure and chemical composition of the Golgi element. Quart. J. Micro. Sci., *85:* 1–71.

———— 1946 The histochemical recognition of lipine. Ibid., *87:* 441–471.

CAIN, A. J. 1947 The use of Nile blue in the examination of lipoids. Quart. J. Micro. Sci., *88:* 383–392.

HOERR, N. L. 1936 Histochemical studies on lipins. I. Osmic acid as a microchemical reagent with special reference to lipins. Anat. Rec., *66:* 149–171.

KAY, W. W., AND R. WHITEHEAD 1937 Fatty substances; from The Microscopist's Vade-Mecum, 10th ed., Chap. 28, pp. 278–285, Blakiston. Philadelphia.

LILLIE, R. D. 1948 Histopathologic technic. Blakiston, Philadelphia.

LISON, L. 1936 Histochimie Animale. Gauthier-Villars, Paris.

MCCLUNG, C. E. 1937 Handbook of microscopical technique. 2nd ed., P. B. Hoeber, Philadelphia.

MONTAGNA, W. 1949 The glands of the external auditory meatus of the cat. J. Morph. (In press.)

MONTAGNA, W., AND C. R. NOBACK 1937 Histochemical observation on the sebaceous glands of the rat. Am. J. Anat., *81:* 39–62.

MONTAGNA, W., AND H. F. PARKS 1948 A histochemical study of the glands of the anal sac of the dog. Anat. Rec., *100:* 297–318.

MONTAGNA, W., AND J. B. HAMILTON 1949 The sebaceous glands of the hamster: some cytochemical studies in normal and experimental animals. Am. J. Anat., *84:* 365–395.

OKEY, R. 1944 Cholesterol injury in the guinea pig. J. Biol. Chem., *156:* 179–190.

PLATE 1

EXPLANATION OF FIGURES

1 Unstained frozen section showing birefringence in the periphery of sebaceous acini. Observe that the sebum, marked *1*, and the mature sebaceous cells, marked *2*, are very weakly anisotropic. The peripheral anisotropic lipids are principally in the form of spherules. × 225.

2 Frozen section treated with Sudan IV. The periphery of the glands is outlined in ink. The sebum and mature sebaceous cells are strongly sudanophilic, but the peripheral cells remain uncolored. × 125.

3 Sebaceous glands treated 10 minutes with Sudan IV and viewed under polarized light. The sebum, marked *1*, and the mature sebaceous cells, marked *2*, show very little birefringence. The peripheral, nonsudanophilic cells, show brilliant birefringence. × 225.

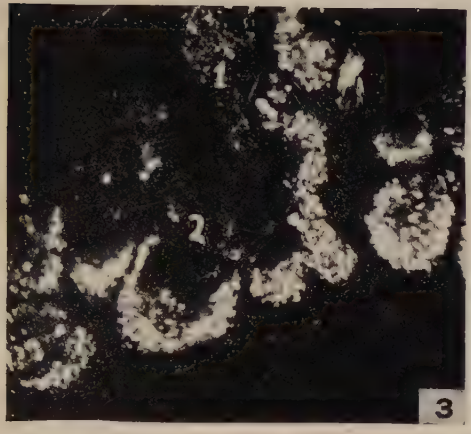
4 Glands treated with Nile-blue sulfate. Birefringence essentially as in untreated section (fig. 1). × 225.

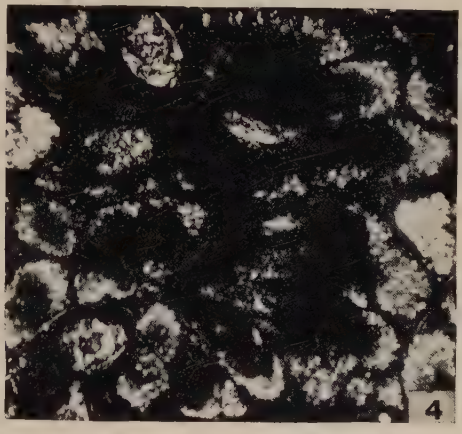
5 Section treated for one minute in Sudan black. There is considerable peripheral birefringence. The spherules are a rose color, the acicular crystals are a light blue. × 225.

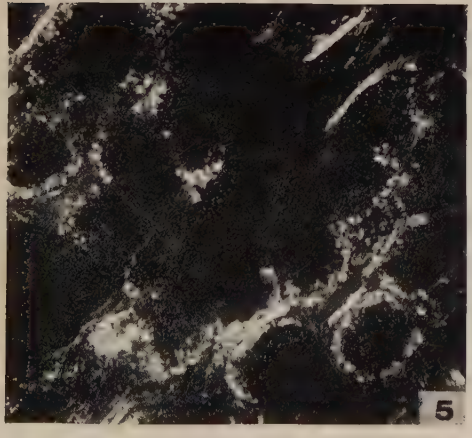
6 Sebaceous gland from unstained frozen sections from the ear of a male cat. The sebum is brilliantly anisotropic, as are also the degenerating and mature sebaceous cells. The white arrows indicate the periphery of the gland which is isotropic. Contrast especially with figure 1. × 225.

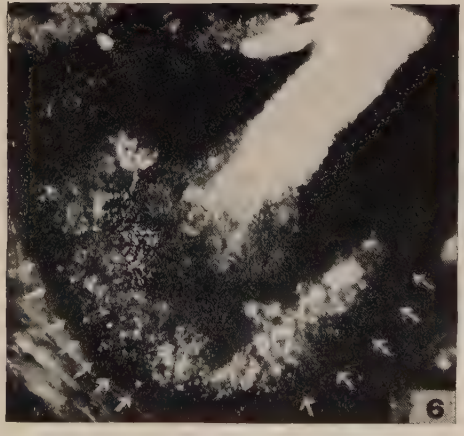
1
2
1

2


1
2
3


4


5


6

THE EFFECT OF THYROXIN INJECTIONS ON GROWTH AND EPIPHYSEAL CLOSURE OF THE THIRD METACARPAL BONE IN HYPOPHYSECTOMIZED FEMALE RATS [1]

C. WILLET ASLING, HERMAN BECKS, MIRIAM E. SIMPSON AND HERBERT M. EVANS

Institute of Experimental Biology, the George Williams Hooper Foundation for Medical Research, the Division of Dental Medicine, and the Division of Anatomy, University of California, Berkeley and San Francisco, California

It has recently been demonstrated that epiphyseal closure occurs in the metacarpal bone of the rat by 110 days of age, in contrast to the delayed union of many of the other epiphyses of this animal (Becks, Asling, Collins, Simpson and Evans, '48). Following hypophysectomy at 28 days of age, this closure fails to take place and the cartilage plate persists into old age, eventually becoming atrophic and sealed from the marrow cavity by bone (Collins, Becks, Asling, Simpson and Evans, '48). The administration of thyroxin for 39 days resulted in complete epiphyseal closure even after post-operative intervals of a year or more (Becks, Asling, Collins, Simpson, Li and Evans, '48). However, the dosage used (7.5 μg/day) proved toxic and had to be reduced during the course of the experiment to 5 μg. The objective of the experiments which are to be described was to repeat the induction of epiphyseal closure, using doses of thyroxin within the tolerance of the rats. Furthermore, it was sought

[1] Aided by grants from the American Cancer Society (through the National Research Council—Committee on Growth), the National Institute of Health, the American Foundation for Dental Science, The California State Dental Association, the Research Board of the University of California, and U. S. Public Health Grant No. RG (800R).

to determine the length of the injection period necessary to cause epiphyseal closure, and to demonstrate any relationship which might exist between this closure and the chronological age of the rat, as well as the length of the bone (i.e., whether the bone was dwarfed or of normal adult length).

Female rats of the Long-Evans strain were hypophysectomized at 27 and at 75 days of age.[2] Following hypophysectomy at the younger of these ages the third metacarpal remained short, reaching an average length of approximately 6.6 mm, or about 87% of the length in the normal adult (7.6 mm). The time of operation for the older of the age-groups was such that the normal adult length had already been attained when hypophysectomy was performed, but epiphyseal closure had not started.

The experimental period for rats hypophysectomized at 27 days of age started at 40 days of age, the post-operative interval of 13 days having been allowed in order to establish provisionally the completeness of hypophysectomy. These rats were divided into three groups, which received the injections for 30, 40, and 71 days, and they were thus 70, 80, and 111 days of age at autopsy. The dose of thyroxin used, 5 μg/day injected 6 days weekly, was well tolerated.

The rats whose skeletal development was more advanced at the time of hypophysectomy (75 days of age) received thyroxin in the same doses as the younger animals, starting the day after operation. No post-operative interval like that in the previous groups could be allowed, since the age of epiphyseal closure was imminent and it was desired to interpose an injection period corresponding in duration to the 30 and 40 day periods employed in the previous group. The injections were started the day after operation, and continued for 35 days. At autopsy these rats were 111 days of age. It will be noted that this age corresponded to the age of the rats undergoing the 71 day injection period above,

[2] The findings reported represent observations on 48 rats hypophysectomized at 28 days and 18 rats hypophysectomized at 75 days of age. Twenty-seven normal rats were included as untreated intact controls.

and to the age at which complete epiphyseal closure is normally found. Control groups of rats, both normal and hypophysectomized, were sacrificed with each of the injected groups. The sella turcica of all hypophysectomized rats was inspected under the binocular dissecting microscope, and no animals were included in the report in which fragments of pituitary tissue were found.

Records were kept of the body weight of all rats during the experiment. The body length (nose to anus and anus to tail tip) was measured at autopsy. The length of the third metacarpal was measured in roentgenograms of the forepaw made (a) at the onset of the injection period, and (b) at autopsy. Histologic sections were made of the metacarpal of all rats to determine the status of the epiphyseal cartilage plate of this bone.

Table 1 shows the arrangement of the animals into groups, the body length and weight at autopsy, the change in body weight during the injection periods, the length of the third metacarpal, and the status of its epiphysis.

The injection of thyroxin for 30 or 40 days in rats hypophysectomized at 27 days of age was accompanied by a slight weight gain which exceeded that of their controls. However, the group receiving injections for the longest period (71 days) did not continue to exceed their controls. In the rats hypophysectomized at 75 days of age the injected and control groups both lost weight slightly.

The length of injected rats hypophysectomized at 27 days of age slightly exceeded their uninjected controls at autopsy, but was far short of normal. After 71 days of injections the injected rats were 76%, and the controls, 69% of normal length. The length of rats hypophysectomized at 75 days of age was not appreciably changed by the injections. At 111 days of age they were slightly shorter than normal—96% and 95% for injected and control groups, respectively.

The length of the metacarpals of injected rats hypophysectomized at 27 days of age significantly exceeded that of their controls by the end of 71 days of injections. The meta-

TABLE 1

The effects of thyroxin on growth and epiphyseal closure of the metacarpal bone of hypophysectomized female rats

AGE			TREATMENT	NO. OF RATS	BODY WEIGHT		LENGTH [1] AT AUTOPSY	THIRD METACARPAL		
								Length		Status of epiphyseal plate [2]
Hypophysectomy	Onset of injection	Autopsy			Autopsy	Gain		Onset	Autopsy	
days	*days*	*days*			*gm*	*gm*	*cm*	*mm*	*mm*	
			None	9	75 ± 2.0	0 ± 1.2	..	6.6 ± 0.07	6.6 ± 0.05	+++++++++
	40	70	Thyroxin 30 days	9	85 ± 2.2	+ 10 ± 1.4	..	6.6 ± 0.07	6.8 ± 0.09	+++++++++
			None	6	77 ± 1.9	+ 3 ± 0.9	25.8 ± 0.23	6.7 ± 0.08	6.7 ± 0.07	++++++
27	40	80	Thyroxin 40 days	8	93 ± 3.5	+ 21 ± 3.5	27.8 ± 0.29	6.5 ± 0.09	6.8 ± 0.07	++++++++
			None	8	86 ± 3.1	+ 18 ± 2.6	26.3 ± 0.33	6.6 ± 0.04	6.7 ± 0.06	++++++++
	40	111	Thyroxin 71 days	8	95 ± 4.3	+ 25 ± 3.3	28.8 ± 0.31	6.5 ± 0.05	7.0 ± 0.04	+ ±———————
			None	7	157 ± 5.5	— 17 ± 4.4	35.7 ± 0.32	7.4 ± 0.04	7.5 ± 0.05	+++ ±±±—
75	76	111	Thyroxin 35 days	11	160 ± 4.2	— 21 ± 2.5	36.3 ± 0.33	7.6 ± 0.07	7.6 ± 0.06	———————————
Normal	..	111	None	10	215 ± 6.0	...	37.9 ± 0.33	...	7.5 ± 0.11	±±———————

[1] Length from nose to anus and anus to tail tip, measured under anesthesia.

[2] Each symbol represents the epiphyseal plate of an individual animal, as follows: + intact, ± perforated, — absent.

carpals of uninjected rats showed no growth during this period.[3] Like the body length, the length of this bone was less than normal in all groups of rats hypophysectomized at this age. At 111 days of age, the metacarpals of injected rats were 92%, and controls, 88% of normal length. The length of the metacarpals of rats hypophysectomized at 75 days of age remained the same both in injected and control groups. It should be remembered that this age of 75 days had been selected as the age for operation because the normal definitive length had already been attained.

Table 1 shows that the injection of thyroxin did not bring about premature epiphyseal closure in rats hypophysectomized at 27 days of age. The cartilage plate was still present after injection for 40 days, at which time the animals were 80 days of age. However, after 71 days of injection at the normal age, epiphyseal closure was found in the majority of rats. The proportion of bones having remnants of the cartilage plate was the same as in normal rats of this age, 111 days. The failure of epiphyseal closure to occur in uninjected rats after hypophysectomy at 27 days of age agrees with previous findings (Collins, Becks, Asling, Simpson, and Evans '48).

When hypophysectomy was performed at 75 days of age, a time at which epiphyseal closure was imminent, the operation delayed but did not prevent epiphyseal closure. At 111 days of age the proportion of epiphyseal plates which remained intact or were perforated was the same as that in normal rats of a slightly younger age, 90 to 100 days. The injection of thyroxin for 35 days resulted in epiphyseal closure in these rats at the normal age, all of them showing complete removal of the epiphyseal cartilage plate at 111 days.

SUMMARY

In keeping with previous experience, epiphyseal closure of the third metacarpal had not occurred at the normal age

[3] They were, however, significantly longer than the average length of this bone at the age of hypophysectomy, 6.3 ± 0.04 mm.

(111 days) in rats hypophysectomized at 27 days of age. Epiphyseal closure was brought about by the normal age, by daily injections of 5 μg of thyroxin for a period of 71 days. Epiphyseal closure did not occur following shorter injection periods (30 days and 40 days), at which times of autopsy the rats were chronologically younger than the normal age of closure for this bone. Although the bones of these injected rats were slightly longer than those of their uninjected hypophysectomized controls, they were shorter than normal.

On the other hand, when hypophysectomy was performed at 75 days of age, a time when normal dimensions had already been attained, epiphyseal closure was delayed, but was in progress at the time of autopsy (111 days) in uninjected rats. In these animals, complete closure was brought about within a 35-day period of injection at the normal chronological age.

LITERATURE CITED

BECKS, H., C. W. ASLING, D. A. COLLINS, M. E. SIMPSON AND H. M. EVANS 1948 Changes with increasing age in the ossification of the third metacarpal of the female rat. Anat. Rec., *100:* 577–591.

BECKS, H., C. W. ASLING, D. A. COLLINS, M. E. SIMPSON, C. H. LI AND H. M. EVANS 1948 Response to pituitary growth hormone and thyroxin of the third metacarpal in the hypophysectomized female rat. Anat. Rec., *101:* 17–22.

COLLINS, D. A., H. BECKS, C. W. ASLING, M. E. SIMPSON AND H. M. EVANS 1948 Changes in the ossification of the third metacarpal at progressively longer intervals following hypophysectomy in the female rat. Anat. Rec., *101:* 13–16.

BOOK REVIEW

THE ANATOMY OF SEMNOPITHECUS ENTELLUS. By A. A. AYER, Professor of Anatomy, Medical College, Madras. Indian Publishing House, Ltd., 1948. 182 pp. $6.00.

The author states in his preface that the book is a record of personal observations on the anatomy of an Old World monkey. As such, it gives evidence of thorough dissection and astute observation, and is well illustrated with the author's drawings. He further states that it is not intended to be a reference book on Primates nor a comprehensive work on the anatomy of the monkeys. Aspects of both functions, however, are included within the scope of the monograph. The bibliography is extensive, as far as 1942. Throughout the descriptions there are references to conditions in other Primates, and comparisons are made among the various species. Hill's ('36) classification of the Old World monkeys has been adopted. This places Semnopithecus in the family Colobidae and subfamily Presbytinae.

The chapters on the muscular system and the facial musculature are well and concisely written, much description being saved by reference to the same structures in man. The chapters dealing with the viscera are especially good. The text of the vascular system, however, could be expanded, and more diagrams would help to interpret the descriptions. In the chapter on the peripheral nervous system, the spinal nerves and plexuses are well described and diagrammed, but only half a page is devoted to the cranial nerves. At the end of the descriptive chapters in the book, the author has placed a summary of the features which he believes have a significance in determining the affinities of Semnopithecus. In the last chapter, he gives his opinion of the extent of these affinities.

It is to be regretted that this work had to wait 6 years for publication. The book is a good contribution to the literature of Primates and furnishes much needed information on the anatomy of an important species.

R. A. Miller